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(54) Title: <b>MODIFIED SFV MOLECULES WHICH MEDIATE ADHESION BETWEEN CELLS AND USES THEREOF</b>					
<p>(57) Abstract</p> <p>The modified sFv molecules of the present invention stimulate adhesion between cells thereby enhancing the immune response against disease. These molecules generally comprise an antigen binding site of an antibody and at least a portion of a transmembrane domain of a cell surface receptor.</p>					
<p>Legend: — HeLa NC3-2 --- HeLa 5B-32 .... HeLa BT-1 .... HeLa</p> <p>C028-Ig</p> <p>Anti-B7-1</p> <p>Anti-Lumen Ig</p> <p>Relative Cell Number</p> <p>Fluorescence Intensity</p>					

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**MODIFIED SFV MOLECULES WHICH MEDIATE ADHESION  
BETWEEN CELLS AND USES THEREOF**

Throughout this application various publications are referenced. The disclosures of these publications in their entireties are hereby incorporated by reference into this application in order to more fully describe the state of the art to which this invention pertains.

5        The present invention relates to modified sFv molecules and uses thereof. The modified sFv molecules of the invention mediate adhesion between cells and comprise a binding site of an antibody and a transmembrane domain of a cell surface receptor.

**BACKGROUND OF THE INVENTION**

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Naive CD4+ T-cells require two independent signals in order to be successfully activated and capable of undergoing clonal expansion (Janeway, *Cold Spring Harbor Symp. Quant. Biol.* 54:1-14 (1989)). The first signal is achieved by stimulation through the T-cell receptor by immunogenic peptides presented by MHC class II molecules on 15 antigen presenting cells (APC) (Weiss, *J. Clin. Invest.* 86:1015 (1990)).

In addition, a second signal, referred to as costimulation, is also required. This costimulatory signal is generally provided through the ligation of CD28 on the T-cell and its inducible counter-receptor CD80, or CD86 on the APC (Linsley et al, *J. Exp. Med.* 173:721-730 (1991)).

20        The modified single chain Fv (sFv) molecules of the invention, when expressed on a cell surface, act as artificial co-stimulatory ligands. They were constructed to enhance an immune response to disease.

Others have constructed sFv molecules for purposes of intracellular targeting to combat disease (Biocca and A. Cattaneo, (1995) *Trends in Cell Biology* 5:248-252).

25        However, the sFv molecules so constructed did not comprise a transmembrane domain which could be anchored to an extracellular surface (Biocca and Cattaneo, *supra*).

sFv molecules are one example of a myriad of molecules that are being tested for potential therapeutic and diagnostic uses against disease. Additional molecules of this type are needed.

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**SUMMARY OF THE INVENTION**

5 The modified sFv molecules of the present invention stimulate adhesion between cells thereby enhancing an immune response against disease. These molecules generally comprise a binding site of an antibody and at least a portion of a transmembrane domain of a cell surface receptor.

In one embodiment of the invention, the modified sFv molecule further comprises a linker which connects the binding site to at least a portion of the transmembrane domain. In a specific embodiment, the modified sFv molecule 10 comprises a binding site which recognizes and binds the CD28 receptor, a Fc portion of an antibody, and at least a portion of a transmembrane domain. In this embodiment, the binding site has two variable regions (V<sub>H</sub> and/or V<sub>L</sub> chains) and the Fc portion connects the binding site with the transmembrane domain.

**15 BRIEF DESCRIPTION OF THE DRAWINGS**

Figure 1 is a FACS analysis of Hela cells infected with retroviral constructs NC3-2 (—), 58-32 (----), B7-1 (also known as CD80) (-----), or non-infected cells (....). Hela cells infected with the 58-32 or NC3-2 retroviruses express the 2E12 sFv on the 20 cell surface as detected either by binding to CD28 or by binding to anti-human Ig. The Hela cells infected with the B7-1 (CD80) retrovirus express high levels of B7-1 (CD80).

Figure 2 is a FACS analysis showing that the GPI anchor from CD58 is cleaved by phospholipase C in the NIH3T3 retrovirus packaging cell line, but is resistant to phospholipase C digestion in the 58-32 Hela cell line. NIH3T3 virus packaging cells 25 (A) or Hela cells (B) infected with retroviral constructs containing the 2E12 sFv-Ig fusion protein linked to a CD58 GPI anchored tail. Surface expression of the 2E12 sFv was analyzed by flow cytometry prior to (—) or following (—) PI-PLC digestion.

Figure 3 is a bar graph showing that Hela cells expressing the anti-CD28 sFv are equivalent to the Hela cells expressing B7-1 (CD80) in their ability to stimulate T cell 30 proliferation. The signal to the T cells is specific for the CD28 receptor, and that the activity is not due to a ligand that binds to CTLA-4, such as B7-1 (CD80) or B7-2 (CD86).

Figure 4 is a line graph showing titration of Hela stimulator cells for induction of proliferation of rested T cell blasts. Hela NC3-2 and Hela 58-32 cells expressing the 35 2E12 sFv on the surface were comparable to the Hela B7-1 (CD80) cells for their ability to induce proliferation.

Figure 5 is a photograph of a gel showing induction of tyrosine phosphorylation of vav protooncogene by Hela cells expressing ligands for human CD28. Hela cells expressing the cell surface 2E12 sFv are equivalent to the Hela cells expressing B7-1 (CD80) for their ability to rapidly activate the tyrosine kinases that phosphorylate the  
5 vav protooncogene.

Figure 6 is diagram of plasmid PLNCX.

Figure 7 is a diagram of plasmid PLNC-2e12hlgG1hB7-1Tm.

Figure 8 is diagram of plasmid PLNC-2e12hlgG1CD58GPI.

Figure 9 is a diagram of plasmid PLNC-hB7-1.

10 Figure 10 provides a nucleic acid sequence of a modified sFv encoded by PLNC-2e12hlgG1hB7-1Tm.

Figure 11 provides a nucleic acid sequence of a modified sFv encoded by PLNC-2e12hlgG1CD58GPI.

15 Figure 12A provides a nucleic acid sequence of the 2E12 sFv having the starting and ending sequences shown.

Figure 12B-D provides nucleic acid and amino acid sequences of the Fc portion of an antibody, namely, human IgG1, having the starting and ending sequences shown.

Figure 12E provides a nucleic acid sequence of the B7-1 transmembrane domain.

20 Figure 12F provides a nucleic acid sequence of CD58 GPI.

Figure 13 provides the nucleotide sequence of the 9.3  $V_L$  and  $V_H$  and the gene fusion created in the sFv. The restriction sites used for subcloning the sFv are shown at each end of the sequence, and the 9.3 native leader sequence for the light chain is boxed and labeled. The complementarity determining regions (CDR) are also boxed  
25 and labeled. The  $V_L$  is most homologous to the murine kappa III V-region family as defined by Kabat et al. (Sequences of proteins of immunological interest. 45th edition. Bethesda, MD; Public Health Service, National Institutes of Health, 1987)) and the  $V$  gene segment has rearranged with a J gene segment homologous to murine Jk2 (underlined and labeled). The  $V_H$  is most homologous to the B subfamily of the  
30 murine I V region family. The heavy chain V gene has rearranged with a J gene segment homologous to murine JH4 (underlined and labeled).

Figure 14 is a photograph of a Western blot of culture supernatants from COS cells. COS cell supernatants (100 ml) from mock transfected cells (Lane 1) L6 sFvIg (Lane 2), 9.3 sFvIg (Lane 3), and 9.3/L6 sFvIg (Lane 4) were immunoprecipitated  
35 with 50  $\mu$ l Staphylococcus protein A beads, washed, boiled in loading buffer, and subjected to SDS-PAGE using 6-15% gradient gels. Gels were blotted to

nitrocellulose and incubated with alkaline phosphatase conjugated goat anti-human IgG to visualize proteins. The L6 and 9.3 sFvIg proteins migrate at  $M_r$  55,000, the 9.3/L6 sFvIg proteins migrate at  $M_r$  83,000, the approximate size expected for these fusion proteins.

5       Figure 15a/b/c are line graphs showing that the bispecific 9.3/L6 sFvIg fusion protein binds to L6<sup>+</sup> H2981 tumor cells and to CD28<sup>+</sup> CHO cells. Panel 15A: L6<sup>+</sup> H2981 tumor cells were incubated with L6 sFvIg (----) at 0.5  $\mu$ g/ml or 9.3 sFvIg (.....) at .5  $\mu$ g/ml or 9.3/L6 sFvIg (\_\_\_\_) fusion protein at 0.5  $\mu$ g/ml or with medium alone (....). Panel 15B: CD28<sup>+</sup> CHO cells were incubated with 9.3 sFvIg (----) at 1  $\mu$ g/ml or 9.3/L6 sFvIg fusion protein (\_\_\_\_) at 1  $\mu$ g/ml, or with medium alone (....). Bound protein was detected with FITC conjugated goat anti-human IgG at 1:100. Panel 15C: CD28<sup>+</sup> PHA blasts were incubated with FITC conjugated L6  $\alpha$ -idiotype mAb 13B (....) as negative control, bispecific 9.3/L6 sFvIg at 0.5  $\mu$ g/ml and bound protein detected with FITC conjugated L6  $\alpha$ -idiotypic mAb 13B (----). Preincubation 10 of cells with unlabelled 9.3 sFvIg inhibited binding of bispecific 9.3/L6 sFvIg as 15 shown by a significant reduction in FITC-13B staining (\_\_\_\_). A total of 10,000 cells were analyzed per sample.

Figure 16 is a bar graph showing 9.3/L6 sFvIg mitogenicity in resting CD28<sup>+</sup> PHA blasts.

20       Figure 17 is a bar graph comparing the abilities of 9.3/L6 sFvIg and 9.3 sFvIg molecules to stimulate T cell proliferation in the presence of tumor cells or crosslinking reagents.

25       Figures 18A-H are line graphs showing expression of CD28 or CD80 on transduced H3347 experimental tumor lines. H3347 tumor cell transfectants were assessed for cell surface expression of the sFvIg or B7 molecules by indirect immunofluorescence using 1:100 FITC anti-human IgG, B-CD28Ig and PE-streptavidin for 9.3 transfectants and 9.3/L6 sFvIg, B-L6 and PE-streptavidin for staining H3347 cells, or B-  $\alpha$ CD80Ig plus PE streptavidin for B7 transfectants. Cells were washed, resuspended in staining media, and analyzed by flow cytometry. A total 30 of 10,000 cells was analyzed for each sample. Transduced cell clones are each identified on the left side of the figure. The first panel of curves shows the fluorescence profile obtained using FITC anti-human IgG on transduced clones with (—) and without (----) FITC label, and the second panel shows staining with the PE-streptavidin conjugate for each transfected clone or sFvIg bound cell, PE-SA alone

(----) and B-molecule indicated plus PE-SA (—). The biotin-labelled reagent used on each clone is identified on the right side of the second panel.

Figure 19 is a bar graph showing a comparison of costimulation by soluble 9.3 sFvIg and soluble CD80Ig. 7-day resting PHA blasts were cocultured for 3 days with 5 dilutions (20 ng/ml, 0.1 µg/ml, 0.5 µg/ml, and 1 µg/ml) of the 9.3 sFvIg or CD80Ig. Stimuli were incubated with no cross-linker anti-human IgG reagent at 4:1 (protein:IgG), or protein A at 4:1 (protein:proteinA). [<sup>3</sup>H]-thymidine incorporation was measured during the last six hours of the assay. Results are displayed as (cpm incorporated x 10<sup>3</sup>). All data are the mean of triplicate samples. SEM is 6% or less.

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### DETAILED DESCRIPTION OF THE INVENTION

#### **DEFINITION**

As used in this application, the following words or phrases have the meanings 15 specified.

As used herein a "modified sFv molecule" is a recombinantly produced antibody fragment comprising a binding site of an antibody and a transmembrane domain of a cell surface receptor or portion thereof. So long as the binding function of the molecule is preserved, the modified sFv molecules can include additional amino acid sequences 20 linked to either its C- or N- terminus and the nucleic acid molecules encoding the modified sFv molecules can include additional nucleotides to its 5' or 3'-terminus.

As used herein a "binding site" means the portion of the molecule which recognizes and binds a target. The binding site includes one or more variable regions.

As used herein "variable region" means a variable heavy (V<sub>H</sub>) chain or a variable 25 light (V<sub>L</sub>) chain, in its entirety or portion thereof which recognizes and binds its target.

As used herein "leucocyte antigen" includes any cell surface receptor having a transmembrane domain and found on a leucocyte.

As used herein a "transmembrane domain" means that portion of a cell surface receptor that anchors the receptor to the membrane or transits a membrane. The 30 transmembrane domain can include a cytoplasmic region (also known as a cytoplasmic tail). The cytoplasmic region may or may not have signaling capabilities, i.e., the capability to interact with cytoplasmic components that are directly or indirectly involved in the transduction of the antigen binding signal.

As used herein "at least a portion of a transmembrane domain" means any 35 portion of the transmembrane domain that serves to anchor the cell surface receptor to

the cell membrane. It is that portion of the transmembrane domain that spans the whole width of a membrane or any part thereof thereby serving to anchor the cell surface receptor to the cell membrane.

As used herein a "linker" means any molecule that links the binding site to the transmembrane domain.

In order that the invention herein described may be more fully understood, the following description is set forth.

## MODIFIED SFV MOLECULES OF THE INVENTION

10

The present invention provides modified sFv molecules which serve as artificial adhesion receptors. These molecules can mediate adhesion between lymphocytes. These molecules can also mediate adhesion between lymphocytes and non-lymphocytic cells.

15

Typically, the modified sFv molecule of the invention comprises a binding site of an antibody and at least a portion of a transmembrane domain of a cell surface receptor. The binding site can have one or more variable regions. Two variable regions are preferable, e.g.,  $V_H$  and  $V_L$  chains.

20

The transmembrane domain can include a cytoplasmic region of a cell surface receptor. The cytoplasmic region can be in its entirety or a portion thereof. It may or may not exhibit signalling capabilities. The cytoplasmic region may be from the same cell surface receptor as the transmembrane domain or from a different cell surface receptor. The cytoplasmic region can contain activation sequences such as antigen receptor homology 1 (ARH1) or enzymatic domains such as protein tyrosine kinase (PTK) or protein tyrosine phosphatase (PTP).

25

In a further embodiment of the invention, the binding site recognizes and binds a first leucocyte antigen. Additionally, the transmembrane domain is from a second leucocyte antigen. The first and second leucocyte antigens may be the same or different. Preferably, the first leucocyte antigen is different from the second leucocyte antigen.

30

Examples of leucocyte antigens include, but are not limited to, CD1, CD2, CD3/TCR, CD4, CD5, T12, CD7, CD8, CD9, CD10, CD11, CD13, CD14, CD15, CD18, CD19, CD20, CD21, CD22, CD23, CD24, CD25, CD26, CD27, CD28, CD29, CD30, CD31, CDw32, CD33, CD34, CD35, CD37, CD38, CD40, CD41, CD43, CD44, CD45, CD46, CD48, CD49, CDw50, CD51, CDw52, CD53, CD54, CD55, CD56, CD57, CD58, CD59, CDw60, CD61, CD62, CD63, CD64, CDw65, CD66, CD67, CD68, CD69, CDw70, CD71, CD72, CD73, CD74, CD75, CD76, CD77, CDw78, 4-

IBB, 4F2, 114/A10, B7-1 (CD80), B7-2 (CD86), B-G, BP-1/6C3, C5aR, c-Kit, CMRF35 antigen, CTLA-4, endoglin, Fas, FcαR, FcεRI, flk-2, fMLPR, G-CSFR, GM-CSFR, gp42, gp49, HSA, ICAM-2, IFNgR, IL1R, IL3R, IL4R, IL5R, IL6R, IL7R, IL8R, LAG-3, LDLR, L-Selectin, Itk, Ly-6, Ly-9, Ly-49, Mac-2, Mannose receptor, M-5 ASGP-BP, M-CSFR, MDR1, MHC Class I, MHC Class II, MIC2, mIgM, MRC OX-2, MRC OX-40, MRC OX-47, NKG2, NKR-P1, PG-1, R2, RT6, Scavenger RI and II, Syndecan, TAPA-1, Thy-1, and TNFRI and II.

In accordance with the practice of the invention, the first or second leucocyte antigen is CD28. Additionally, the first or second leucocyte antigen is B7 (CD80 or 10 CD86). Further, the first or second leucocyte antigen is CTLA4.

In accordance with the practice of the invention, the modified sFv molecule may further comprise a linker. The linker connects the binding site to at least a portion of a transmembrane domain. Additionally, the linker can be useful as an identification tag for detection purposes.

15 A Fc portion of an antibody is one example of a linker. Another examples of a suitable linker is a helical peptide linker (Newton CR, et al., Cloning and expression in murine erythroleukemia cells: the soluble forms type I and type II tumor necrosis factor receptors fused to an immunogenic affinity tag. *Protein Expression and Purification*, 1994 Oct, 5(5):449-57). Additionally, The "tag" peptide 20 -Ala-Ala-Asn-Asp-Glu-Asn-Tyr-Ala-Leu-Ala-Ala-COOH is another example of a linker (Tu GF, et al, C-terminal extension of truncated recombinant proteins in *Escherichia* 10Sa RNA decapeptide. *Journal of Biological Chemistry*, 1995 Apr 21, 270(16):9322-6).

25 Another suitable linker is the hinge-like region of B7-1 or B7-2. A peptide segment or a second functional domain such as an Ig, a growth hormone, an adhesion receptor, or another sFv or part thereof are examples of suitable linkers.

Further, the FLAG sequence DYKDDDDK is an example of a linker (Knappik A; Pluckthun A., An improved affinity tag based on the FLAG peptide for the detection and of recombinant antibody fragments. *Biotechniques*, 1994 Oct, 17(4):754-61).

30 Further, another example of a linker is the Flag peptide consisting of the 11-amino-acid leader peptide of the gene product from bacteriophage T7 (Witzgall R, et al, A mammalian expression vector for the expression of GAL4 fusion proteins with an epitope tag and histidine tail. *Analytical Biochemistry*, 1994 Dec, 223(2):291-8).

35 The "Strep tag" is yet another example of a linker (Schmidt TG; Skerra A., One-step affinity purification of bacterially produced proteins by means of the "Strep

tag" and immobilized recombinant core streptavidin. *Journal of Chromatography*. a, 1994 Aug 5, 676(2):337-45).

The influenza virus hemagglutinin (HA) epitope tag is an example of a linker (Chen YT, et al, Expression and localization of two low molecular weight GTP-binding 5 proteins, Rab8 and Rab10, by epitope tag. *PNAS*, 1993 Jul 15, 90(14):6508-12).

A 14-amino acid oligopeptide in simian virus 5(SV5) is still another example of a linker (Hanke T, et al, Construction of solid matrix-antibody-antigen complexes containing simian immunodeficiency virus p27 using tag-specific monoclonal antibody and tag-linked antigen. *Journal of General Virology*, 1992 Mar, 73 (Pt 3):653-60).

10 Also, the sequence Ala-Leu-Ala-Leu is an example of a linker (Studer M, et al, Influence of a peptide linker on biodistribution and metabolism of antibody-conjugated benzyl-EDTA. Comparison of enzymatic digestion in vitro and in vivo. *Bioconjugate Chemistry*, 1992 Sep-Oct, 3(5):424-9).

15 The myc epitope is another example of a linker (Simons M, et al, Intracellular routing of human amyloid protein precursor: axonal delivery transport to the dendrites. *Journal of Neuroscience Research*, 1995 May 1, 41(1):121-8).

16 The seven-histidine tag is another example of a linker (Parks TD, et al, Expression and purification of a recombinant tobacco etch virus N1a proteinase: biochemical analyses of the full-length and a naturally occurring truncated proteinase 20 form. *Virology*, 1995 Jun 20, 210(1):194-201; Vouret-Craviari V, et al, Post-translational and activation-dependent modifications of the G protein-coupled thrombin receptor. *Journal of Biological Chemistry*, 1995 Apr 7, 270(14):8367-72).

25 The synthetic peptide based on the amino acid sequence of the C terminal region of native human factor X activation peptide (FXAP) is another example of a linker (Philippou H, et al, An ELISA for factor X activation peptide: application to the investigation of thrombogenesis in cardiopulmonary bypass. *British Journal of Haematology*, 1995 Jun, 90(2):432-7).

30 Another linker is the small antigenic peptide epitope (YPYDVPDYAIEGR) containing part of the hemagglutinin (HA) of influenza virus (Kast C, et al, Membrane topology of P-glycoprotein as determined by epitope insertion: transmembrane organization of the N-terminal domain of mdr3. *Biochemistry*, 1995 Apr 4, 34(13):4402-11).

35 The epsilon-tag peptide is another linker (Lehel C, et al, Protein kinase C epsilon is localized to the Golgi via its zinc-finger modulates Golgi function. *PNAS*, 1995 Feb 28, 92(5):1406-10).

The KGF-SYFGEDLMP peptide is another linker. This sequence is derived and is encoded by the tagging insert sequence of Olah Z, et al, A cloning and epsilon-epitope-tagging insert for the expression of polymerase chain reaction-generated cDNA fragments in *Escherichia coli* and mammalian cells. *Analytical Biochemistry*, 5 1994 Aug 15, 221(1):94-102.

Another linker is the six histidine tag (Sporeno E, et al, Production and structural characterization of amino terminally histidine tagged human oncostatin M in *E. coli*. *Cytokine*, 1994 May, 6(3):255-64).

The streptavidin-affinity tag is also an example of a linker (Schmidt TG; Skerra 10 A., The random peptide library-assisted engineering of a C-terminal affinity peptide, useful for the detection and purification of a functional Ig Fv fragment. *Protein Engineering*, 1993 Jan, 6(1):109-22).

The hemagglutinin epitope sequence, YPYDVPDYA (HA1) is yet another 15 example of a linker (Pati UK., Novel vectors for expression of cDNA encoding epitope-tagged proteins in mammalian cells. *Gene*, 1992 May 15, 114(2):285-8).

Preferably, the linker should not exhibit cross reactivity with the binding site for the ligand. Further, the linker should not recognize and bind the binding site. For example when the binding site recognizes and binds the CD28 receptor, the linker cannot be the CD28 receptor or parts thereof.

20 The linker may provide structural support, functional support, or both. For example, the Fc linker provides effector functions as well as structural function, i.e., in connecting the binding site of the molecule to the transmembrane domain.

One example of the invention includes a modified sFv molecule comprising a 25 binding site of an antibody which recognizes and binds the CD28 receptor (e.g., the 2E12 sFv (shown in Figure 12A) or the binding site of the 9.3 antibody), a Fc portion of an antibody, and at least a portion of a transmembrane domain and cytoplasmic tail of the B7 receptor. Like 2E12 sFv, the 9.3 monoclonal antibody (mAb) recognizes and binds the CD28 receptor (ATCC No. HB 10271, Hansen et al., *Immunogenetics* 10:247-260 (1980); Parham, et al. *J. Immunol.* 131:2895-2902 (1983)).

30 In comparison with CD80, a natural adhesion receptor for CD28, the 2E12 sFv showed increased binding affinity for CD28 and was efficient in activating T cells during co-culture. Because of these characteristics, the cell surface expression of modified sFv molecules offer advantages over natural ligands for binding and activation of adhesion receptors.

35 In another embodiment, the invention provides a modified sFv molecule comprising a binding site of an antibody which recognizes and binds the CD28 receptor,

a Fc portion of an antibody, and at least a portion of a transmembrane domain which is a CD58 GPI tail.

Other modifications to the sFv molecules of the invention are possible. These modifications include the addition of protein or peptide segments or modification of existing segments which would enhance the molecules' ability to mediate adhesion between cells. Additionally, these modifications involve amino acid substitutions within the molecule. These substitutions include, but are not necessarily limited to, amino acid substitutions known in the art as "conservative".

For example, it is a well-established principle of protein chemistry that certain amino acid substitutions, entitled "conservative amino acid substitutions," can frequently be made in a protein without altering either the conformation or the function of the protein. Such changes include substituting any of isoleucine (I), valine (V), and leucine (L) for any other of these hydrophobic amino acids; aspartic acid (D) for glutamic acid (E) and vice versa; glutamine (Q) for asparagine (N) and vice versa; and serine (S) for threonine (T) and vice versa.

Other substitutions can also be considered conservative, depending on the environment of the particular amino acid and its role in the three-dimensional structure of the protein. For example, glycine (G) and alanine (A) can frequently be interchangeable, as can alanine and valine (V).

Methionine (M), which is relatively hydrophobic, can frequently be interchanged with leucine and isoleucine, and sometimes with valine. Lysine (K) and arginine (R) are frequently interchangeable in locations in which the significant feature of the amino acid residue is its charge and the differing pK's of these two amino acid residues are not significant. Still other changes can be considered "conservative" in particular environments.

#### NUCLEIC ACID MOLECULES, VECTORS AND HOST VECTOR SYSTEMS FOR MAKING THE MODIFIED SFV MOLECULES OF THE INVENTION

The present invention also provides nucleic acid molecules (such as DNA molecules) encoding the modified sFv molecules of the invention. In one example, the DNA is cDNA having the sequence shown in Figure 10. Additionally, the cDNA has the sequence shown in Figure 11. Figure 12A shows an example of the 5'-portion of a nucleic acid molecule according to the present invention, encoding 2E12. Figures 12B-12D show the nucleic acid sequence of the central portion of a nucleic acid molecule according to the present invention, encoding a portion of IgG1; this portion acts as a

linker. Figure 12E shows the nucleic acid sequence of the 3'-portion of a nucleic acid molecule according to the present invention, encoding the transmembrane domain of B7-1 (CD80). In another alternative, Figure 12F shows the nucleic acid sequence of GPI, which can be used as the nucleic acid sequence of the 3'-portion of a nucleic acid 5 molecule according to the present invention.

Nucleic acid molecules include both DNA and RNA unless otherwise indicated, and can include both single and double-stranded nucleic acid sequences. If a DNA sequence is referred to, reference is generally to both strands of a DNA sequence, either individually or as a Watson-Crick double helix. If only one strand is specified, the 10 complementary strand whose antiparallel sequence is determined by Watson-Crick base pairing rules is also included unless the complementary sequence is specifically excluded. If only one strand is specified in double-stranded DNA, the strand specified is the sense strand, and is the strand that would be equivalent to the sequence of any RNA transcribed from the double-stranded DNA, except for the replacement of thymidine (T) 15 in the DNA by uridine (U) in the RNA. Reference to a nucleic acid sequence also includes modified bases as long as the modification does not significantly interfere with Watson-Crick base pairing or other specified functions of the nucleic acid, and can, for example, include substitution of uridine for thymidine in DNA as well as methylation of bases or modification of sugars.

20 Further, producer cells transfected with such nucleic acid molecules are provided.

The subject invention further provides an expression vector encoding the sFv molecule of the invention. In one embodiment, the expression vector is designated pLNC-2e12-hIgG1-hB7-1Tm (Figure 7). Alternatively, the expression vector is 25 designated pLNC-2e12-hIgG1-hCD58GPI (Figure 8).

The invention further provides a eucaryotic cell transfected with the expression vector of the invention. In one example, the eucaryotic cell is a mammalian cell. Examples include but are not limited to Hela cells, NIH 3T3 cells, and tumor cells such as H334-7 (L6+) and H3396 (BR96+).

30

#### **METHODS OF USING THE MODIFIED SFV MOLECULES AND VECTORS ENCODING THEM**

The invention also provides methods for producing the modified sFv molecules of the invention. In one embodiment, the method comprises culturing the cells 35 transfected by the expression vector of the invention so as to produce the modified sFv molecules and recovering the molecules so produced.

In another embodiment, the invention provides a method for producing modified sFv molecules in a mammalian cell. This method comprises transfecting the mammalian cell with the expression vector of the invention; culturing the mammalian cell so transfected; and recovering the modified sFv molecules so produced by the cultured mammalian cell.

In accordance with the practice of the invention, the step of recovering the biologically active sFv molecule comprises (a) identifying the modified sFv molecule (e.g., by the presence of the binding site or the transmembrane domain); and (b) separating the modified sFv molecule so identified from non-identified molecules, so as to recover the modified sFv molecule so produced by the cultured mammalian cell.

The present invention further provides a method for enhancing a T cell response in a subject. The methods include an ex vivo protocol and an in vivo protocol.

In one embodiment of the ex vivo protocol, the method comprises administering autologous donor cells (e.g., peripheral blood leukocytes (PBLs)) into the subject. In this embodiment, the PBLs are incubated in vitro with the mammalian cells of the invention. The mammalian cells are genetically modified to express modified sFv molecules to its cell surface thereby providing a co-stimulatory molecule for the PBLs. Incubation lasts for a sufficient time for the PBLs to stimulate a T cell response. Post-incubation, the activated PBLs are administered to the subject.

In another embodiment of the ex vivo protocol, the cells can be autologous or allogeneic cells, e.g., PBLs, tumor cells, or tumor infiltrating lymphocytes (TILs).

Allogeneic cells can be encapsulated in order to prevent or inhibit an immune response. Alternatively, the allogeneic cells can be irradiated. Further alternatively, the subject may be administered with an immunosuppressant agent to prevent or inhibit an immune response.

These cells, whether autologous or allogeneic, are genetically modified by insertion of the expression vector of the invention into the cells so as to produce modified sFv molecules attached to the cell surface in a sufficient amount so as to stimulate a T cell response once in the subject and thereby inhibiting tumor growth in the subject.

In one embodiment of the in vivo protocol, the method comprises introducing nucleic acid molecules of the invention or transfection vehicles containing such nucleic acid molecules e.g., a vector, into a producer cell of the invention. Various methodologies for introducing the vector into the producer cells and further examples of transfection vehicles are found infra.

The vector contains DNA encoding the modified sFv molecule of the invention and at least one gene required for replication of the retrovirus into the genome of the producer cells.

In this in vivo protocol, the method further comprises the step of selecting 5 producer cells in which the modified retrovirus is incorporated as part of the genome of the producer cells. The producer cells so selected are then administered in proximity to the tumor cells in order to infect the tumor cells with the modified vector being produced by the producer cells, thereby transferring the DNA to the tumor cells.

In accordance with the practice of the invention, the subject may be an animal 10 subject such as a human, a dog, a cat, a sheep, a horse, a fish, a bird, a pig, or a cow.

Presently, several groups are using gene therapy in cancer treatment (Friedmann T. Progress toward human gene therapy. *Science* 1989; 244:1275-1281; Roth JA, et al. Molecular approaches to prevention and therapy of aerodigestive tract cancers. Review article. *Monogr Natl Cancer Inst* 1992; 13:15-21; Mukhopadhyay T, et al. Specific 15 inhibition of K-ras expression and tumorigenicity of lung cancer cells by antisense RNA. *Cancer Res* 1991; 51:1744-1748).

The most effective mode of administration and dosage regimen for the molecules of the present invention depends upon the location of the tissue or disease being treated, the severity and course of the medical disorder, the subject's health and 20 response to treatment and the judgment of the treating physician. Accordingly, the dosages of the molecules should be titrated to the individual subject.

The interrelationship of dosages for animals of various sizes and species and humans based on  $\mu\text{g}/\text{m}^2$  of surface area is described by Freireich, E.J., et al. *Cancer Chemother., Rep.* 50 (4): 219-244 (1966). Adjustments in the dosage regimen may be 25 made to optimize the tumor cell growth inhibiting and killing response, e.g., doses may be divided and administered on a daily basis or the dose reduced proportionally depending upon the situation (e.g., several divided doses may be administered daily or proportionally reduced depending on the specific therapeutic situation).

It would be clear that the dose of the composition of the invention required to 30 achieve cures may be further reduced with schedule optimization.

## INTRODUCTION OF VECTORS OR NUCLEIC ACID MOLECULES OF THE INVENTION INTO CELLS

A variety of techniques are available for the introduction of nucleic acid 35 molecules into cells. For example, the nucleic acid molecule may be inserted into a cell directly in a recombinant viral vector. Other insertion methods are possible.

For example, in ex vivo techniques, the gene can be inserted into a cell using any gene transfer procedure such as calcium phosphate mediated transfection, the use of polycations or lipids complexed with DNA, encapsulation of DNA in lipid vesicles or erythrocyte ghosts, or the exposure of cells to rapid pulses of high voltage electric current (i.e., electroporation).

5 DNA has also been introduced into cells by direct microinjection or by the use of high-velocity tungsten microprojectiles. These techniques are capable of integrating multiple copies of DNA into the genome although the efficiency of the integration varies widely with the technique, different genes, and different cell types.

10 Recently techniques have been developed using viral vectors to introduce DNA into mammalian cells. These techniques have the potential for infecting all cells exposed to the virus. In developing techniques for the use of viral vectors, it was necessary to develop vectors that stably incorporated into the target cell without damaging it.

15 Suitable viral vectors include papovaviruses, simian virus 40, polyomavirus, adenoviruses, murine and avian retroviruses. Viral vectors can infect multiple cell types.

20 Compared to vectors that do not enter cells by receptor mediated events, viral vectors are preferred because of their efficiency. Examples of suitable viral vectors include, but are not limited to, a retrovirus vector, an adenovirus vector, a vaccinia virus vector, a herpes virus vector, or a rabies virus vector.

25 The viral vector selected should meet the following criteria: 1) the vector must be able to infect the cells of interest and thus viral vectors having an appropriate host range must be selected; 2) the transferred gene should be capable of persisting and being expressed in a cell for an extended period of time; and 3) the vector should be safe to the host and cause minimal cell transformation. Retroviral vectors and adenoviruses offer an efficient, useful, and presently the best-characterized means of introducing and expressing foreign genes efficiently in mammalian cells.

30 These vectors have very broad host and cell type ranges, express genes stably and efficiently. The safety of these vectors has been proved by many research groups. In fact many are in clinical trials.

35 Other virus vectors that may be used for gene transfer into cells for correction of disorders include herpes virus papovaviruses such as JC, SV40, polyoma; Epstein-Barr Virus (EBV); papilloma viruses, e.g. bovine papilloma virus type I (BPV); poliovirus and other human and animal viruses.

Adenoviruses have several properties that make them attractive as cloning vehicles (Bachettis et al.: Transfer of gene for thymidine kinase-deficient human cells by purified herpes simplex viral DNA. PNAS USA, 1977 74:1590; Berkner, K.L.: Development of adenovirus vectors for expression of heterologous genes.

5 Biotechniques, 1988 6:616; Ghosh-Choudhury G, et al., Human adenovirus cloning vectors based on infectious bacterial plasmids: Gene 1986; 50:161; Hag-Ahmand Y, et al., Development of a helper-independent human adenovirus vector and its use in the transfer of the herpes simplex virus thymidine kinase gene. J Virol 1986; 57:257; Rosenfeld M, et al., Adenovirus-mediated transfer of a recombinant  $\alpha_1$ -antitrypsin gene

10 to the lung epithelium in vivo. Science 1991; 252:431).

For example, adenoviruses possess an intermediate sized genome that replicates in cellular nuclei; many serotypes are clinically innocuous; adenovirus genomes appear to be stable despite insertion of foreign genes; foreign genes appear to be maintained without loss or rearrangement; and adenoviruses can be used as high level transient

15 expression vectors with an expression period of weeks to several months. Extensive biochemical and genetic studies suggest that it is possible to substitute up to 7-7.5 kb of heterologous sequences for native adenovirus sequences generating viable, conditional, helper-independent vectors (Kaufman R.J.; Identification of the component necessary for adenovirus translational control and their utilization in cDNA expression vectors.

20 PNAS USA, 1985 82:689).

AAV is a small human parvovirus with a single stranded DNA genome of approximately 5 kb. This virus can be propagated as an integrated provirus in several human cell types. AAV vectors have several advantage for human gene therapy. For example, they are trophic for human cells but can also infect other mammalian cells;

25 (2) no disease has been associated with AAV in humans or other animals; (3) integrated AAV genomes appear stable in their host cells; (4) there is no evidence that integration of AAV alters expression of host genes or promoters or promotes their rearrangement; (5) introduced genes can be rescued from the host cell by infection with a helper virus such as adenovirus.

30 HSV-1 vector system facilitates introduction of virtually any gene into non-mitotic cells (Geller et al. An efficient deletion mutant packaging system for a defective herpes simplex virus vectors: Potential applications to human gene therapy and neuronal physiology. PNAS USA, 1990 87:8950).

Another vector for mammalian gene transfer is the bovine papilloma virus-based

35 vector (Sarver N, et al., Bovine papilloma virus DNA: A novel eukaryotic cloning vector. Mol Cell Biol 1981; 1:486).

Vaccinia and other poxvirus-based vectors provide a mammalian gene transfer system. Vaccinia virus is a large double-stranded DNA virus of 120 kilodaltons (kd) genomic size (Panicali D, et al., Construction of poxvirus as cloning vectors: Insertion of the thymidine kinase gene from herpes simplex virus into the DNA of infectious 5 vaccine virus. *Proc Natl Acad Sci USA* 1982; 79:4927; Smith et al. infectious vaccinia virus recombinants that express hepatitis B virus surface antigens. *Nature*, 1983 302:490.)

Retroviruses efficiently insert viral genes into host cells (Guild B, et al., Development of retrovirus vectors useful for expressing genes in cultured murine 10 embryonic cells and hematopoietic cells in vivo. *J Virol* 1988; 62:795; Hock RA, et al., Retrovirus mediated transfer and expression of drug resistance genes in human hemopoietic progenitor cells. *Nature* 1986; 320:275; Kriegler M. Gene transfer and expression. A laboratory manual. New York: Stockton Press, 1990:1-242; Gilboa E, Eglitis MA, Kantoff PW, et al. Transfer and expression of cloned genes using retroviral 15 vectors. *Biotechniques* 1986; 4:504-512; Eglitis AM, Anderson WF. Retroviral vectors for introduction of genes into mammalian cells. *Biotechniques* 1988; 6:608-614; Adam MA, Miller AD. Identification of a signal in a murine retrovirus that is sufficient for packaging of nonretroviral RNA into virions. *J Virol* 1988; 62:3802-3806; Armentano D, Yu SF, Kantoff PW, et al. Effect of internal viral sequences on the utility of 20 retroviral vectors. *J Virol* 1987; 61:1647-1650; Bender MA, Palmer TD, Gelinas RE, et al. Evidence that the packaging signal of Moloney murine leukemia virus extends into the gag region. *J Virol* 1987; 61:1639-1646; Danos O, Mulligan RC. Safe and efficient generation of recombinant retroviruses with amphotropic and ecotropic host ranges. *Proc Natl Acad Sci USA* 1988; 85:6460-6464; Markowitz D, Goff S, Bank A. 25 Construction and use of a safe and efficient amphotropic packaging cell line. *Virol* 1989; 167:400-406; Miller AD, Buttimore C. Redesign of retrovirus packaging cell lines to avoid recombination leading to helper virus production. *Mol Cell Biol* 1986; 6:2895-2902; Miller AD, Trauber DR, Buttimore C. Factors involved in the production of helper virus-free retrovirus vectors. *Somatic Cell Mol Genet* 1986; 12:175-183; 30 Miller AD, Rosman GJ. Improved retroviral vectors for gene transfer and expression. *Biotechniques* 1989; 7:980-986).

#### ADVANTAGES OF THE INVENTION

The discovery herein lies in modifying sFv molecules by connecting a 35 transmembrane domain to the antigen binding site of the molecule. This modification

creates molecules, namely, artificial ligands, that can further enhance co-stimulatory activity during an immune response.

For example, tumor cells are not immunogenic when they do not express natural ligands (CD80 or CD86) for CD28. Therefore, the molecules of the invention act as 5 artificial adhesion receptors thereby increasing the immunogenicity of tumor cells by increasing the expression of co-stimulatory molecules (such as CD80 or CD86).

This artificial ligand may have several potential advantages over the use of CD80 or CD86. For example, an sFv can have a higher binding affinity than the natural ligand, and therefore may generate a stronger signal. The transmembrane domain can 10 also be chosen to maximize mobility on the cell surface, resulting in a CD28 ligand that has a higher potency than CD80 or CD86.

Further, previous studies have shown that CD28 and CTLA-4 can bind to both CD80 and CD86 and that under some conditions, the signal generated through CTLA-4 binding can be inhibitory. In contrast, cells transfected with an anti-CD28 sFv would 15 only bind through CD28 and the inhibitory effects of CTLA-4 ligation would be eliminated.

The data herein shows that tumor recognition by T cells requires costimulatory signals through adhesion receptors, and suggests that the increased expression of certain leucocyte antigens (e.g., ligands which recognize and binds CD28) on the cell surface 20 may be a desirable goal for tumor gene therapy.

The following examples are presented to illustrate the present invention and to assist one of ordinary skill in making and using the same. The examples are not intended in any way to otherwise limit the scope of the invention.

## 25 EXAMPLE 1

The mAb 1TE+ 19.8.2 2E12 was generated by R.S. Mittler (see Figure 12A).

**Construction of sFv's:** Isolation of variable regions: RNA was isolated from 30 hybridoma 2E12 Using Stratagene mRNA Isolation Kit (Stratagene, Torrey Pines) according to manufacturer's instructions. First strand synthesis was carried out with Stratagene First Strand Synthesis Kit and primers specific for isotype and constant region.

cDNA reactions were poly-G tailed using dGTP and terminal transferase (Stratagene, Torrey Pines), and G-tailed cDNA was used in PCR with a forward primer 35 containing poly-C sequences and reverse primers specific for mouse variable region

(primers contained restriction sites for cloning). PCR products were ligated into PUC19 and positive clones checked by DNA sequencing.

sFv Assembly: Variable light and heavy regions were joined in a single coding region by a (Gly<sub>4</sub>Ser)<sub>3</sub> linker created by using overlap extension PCR. The VL-VH cassette was subsequently ligated into a modified PUC19 vector containing a mutant form of human IgG1-Fc, for use in purification and diagnostic analysis. Finally, the sFv-Ig construct was inserted into an adapted expression vector (CDM8) containing the anti-L6 immunoglobulin light chain leader sequence for secretion of fusion proteins. DNA from positive clones was used in transient COS transfections and supernatants were tested by FACS and Western blot analysis.

**Construction of the retroviral vector pLNC-2e12-hIgG1-hB7-1Tm:** The 2E12 sFv was obtained from R. Mittler as plasmid in MC1061p3 cells. The restriction map was verified and the 2E12 sFv DNA was amplified as a Hind-III/Bgl-II fragment using the primers S2e12SFV-3: (5'-GTGAATTCCAAGCTTCCACC ATG GAT TTT CAA GTG CAG ATT-3' and A2e12SFV: 5'-A GTG CAG ATC TGA GGA GAC GGT GAC-3'. The indicated triplets correspond to open reading frames. The bases in boldface in A2e12SFV indicate the base changes introduced to convert the Bcl-I restriction site at the end of the V<sub>H</sub> domain of the original 2E12 sFv to a Bgl-II site.

PCR reactions were 30 cycles at: 94 C, 30 sec., 48 C, 1 min., 72 C, 3 min. After cleavage with Hind-III and Bgl-II and purification using a QIA-Quick cartridge, the fragment was ligated into the corresponding restriction sites in the pCDNA1-hIgG1(Fc-) vector. The resulting pCDNA1-2e12sFv-hIgG1(Fc-) construct was tested for production of transient expression of the soluble sFv-Ig by transfection into Cos cells.

Cells were stained with CD28mIg followed by goat-anti-mouse Ig FITC conjugate or with goat-anti-human IgFITC conjugate to verify the presence of the sFv and the human Ig domain respectively. Ten of ten tested clones were positive for both but negative for staining with CTLA4mIg, demonstrating that the original CTLA4 insert in the vector had been replaced by a functional 2E12 sFv.

Secretion of the 2e12sFv-hIgG1(Fc-) fusion protein was demonstrated by adsorption of cell culture supernate from transiently transfected cos cells on Protein A Sepharose, followed by SDS-gel electrophoresis. Immunoblotting with CD28mIg at 2.5 µg/ml followed by goat anti-mouse HRP conjugate at 1/10000 dilution demonstrated a weak signal at approx. 57 kd in all tested culture supernates from clones of the 2e12-hIgG1(Fc-) constructs. Clones e7 and e8 were used for further constructions.

For construction of subsequent transmembrane-anchored sFv constructs, the 2e12-hIgG1(Fc-) construct was prepared by PCR from the pCDNA-1hIgG1 vector as a Hind-III/BamH-I fragment with an open reading frame extending through the BamH-I site. The PCR primers used were S2e12SFV-3 (see above) and AIGG1BAM: 5'-CTG 5 CAT CCG GAT CCG CTT TAC CCG GAG ACA GGG AGA GGC-3'. PCR was performed by 30 cycles at: 94 C, 30 sec., 72 C, 5 min.

The Hind-III and BamH-I cleaved PCR fragment was purified as above and ligated into the Hind-III and BamH-I cleaved vector pSFVDNA1. This vector is derived from pCDNA-1 by insertion into the Hind-III and Xba-I sites of a double 10 stranded synthetic 77-mer, which creates the new sequence (from Hind-III to XbaI): 5'- a agc tGA ATT CCA AGC TTG CAT CAG ATC TCT CAT CTA GAG GTT CGG ATC CTT CGA ACC GCA GTC TCG AGC ATC GAT AGc tag a-3'. Lower case letters indicate bases from pCDNA-1.

This cloning linker destroys the flanking Hind-III and Xba-I sites, and 15 introduces the following restriction sites: Eco-RI, Hind-III, Bgl-II, Xba-I, BamH-I, BstB-I, Xho-I and Cla-I and the amino acid (aa) sequence (from EcoR-I to Cla-I): NSKLASDLSSRGSDPSNRSLEHR-stop. Six pSFVDNA-1 clones of the Hind-III - BamH-I 2e12-hIgG1 (Fc-) fragment were tested for transient expression in Cos cells and for secretion of the fusion protein.

20 Clones 2V5, 7, 9, 11, 13 were positive in both tests, but clone 2V1 was negative in both. This fusion protein has the additional aa DPSNRSLEHR attached to the C-terminus (from the BamH-I cloning site).

Next, the transmembrane and cytoplasmic domains from the human B7-1 (CD80) were attached at the BstB-I site 3' of the 2e12-Ig fusion protein gene in 25 pSFVDNA-1. The B7-1 gene fragment was amplified from cDNA using the primers SHB71TM: 5'-CTGCATCCGTTCTG AAC CTG CTC CCA TCC TGG GCC A-3' and AHB71TM: 5'-CAGCGTTGACTCGAGTATCGATTAA TAC AGG GCG TAC ACT TTC CCT T-3'.

30 Amplification conditions were 30 cycles at 94 C, 30 sec., 68 C, 1 min., 72 C, 2 min. The PCR product comprises aa 242-288 of human B7-1, on a 179 bp fragment. After cleavage with BstB-I and Xho-I the 154 bp fragment remaining was purified and ligated into BstB-I and Xho-I cleaved pSFVDNA-1 containing the 2e12-hIgG1(Fc-) (pooled 2V1, 5, 7, 9, 11, 13 as described above).

35 Four clones with the correct 154bp insert were screened for expression in Cos cells of protein reacting with goat anti-mouse Ig and CD28-mIg reagents. Two of these were positive in both tests (2VB7#5 and #12), while two were negative in both tests

(2VB7#3 and #9). Clone pSFVDNA-1 2e12-hIgG1(Fc-)-hB7-1Tm 2VB7#5 was used for the subsequent subcloning of the transmembrane anchored sFv into a retrovirus vector.

Initially three different retroviral vectors were tested for sFv expression levels in 5 transient transfections in Cos cells. Only one vector produced satisfactory expression levels of protein, i.e. pLNCX (Figure 6) (Miller and Rosman (1989) BioTechniques 7:980-990). A purified Hind-III/Cla-I fragment from clone 2VB7#5 was ligated into the Hind-III/Cla-I cloning sites of pLNCX.

Six clones (pLNC-2VB75#1-6) were tested for expression by transient 10 transfection into Cos cells. At 48h post transfection, surface expression of the sFv was tested. The cells on microscope slides were fixed in 2% formaldehyde in PBS for 15 min at RT and then washed briefly with PBS. No detergent permeabilization was used. Non-specific epitopes were blocked for 30 min. at RT in 2% BSA, 10% normal goat 15 serum (NGS) in PBS w. Ca and  $\mu$ g. Primary reagent was 8ml/ $\mu$ g CD28-mlg in 10% NGS, PBS w. Ca and  $\mu$ g for 60 min. at RT. Secondary antibody was goat anti-mouse Ig-FITC at a 1/100 dil. (TAGO, approx. 1  $\mu$ g/ml in 10% NGS, PBS w. Ca and  $\mu$ g).

Clones #2-6 were positive, while clone #1 was negative as expected (wrong insert). Clone pLNC-2VB75#3 (5NC3) was used for all subsequent studies.

20 **Production of packaging cell lines producing retroviruses transducing the 2e12-hIgG1(Fc-)-hB7-1Tm construct and isolation of expressing clones of the human carcinoma lines Hela, H3347 and 2987.** Twenty  $\mu$ g of pLNC-2VB75#3 DNA was transfected on a 10cm plate of the ecotropic packaging cell line PES01 by calcium 25 phosphate precipitation. After 16 h incubation, the medium was replaced (DME, 10% FBS) and transiently produced retrovirus was recovered in the culture medium after a further 24 h. incubation.

30 The virus-containing medium (10ml) was filtered through a 0.2 mm Gelman Acrodisc syringe filter. Hexadimethrine bromide was added to a final 8  $\mu$ g/ml and the medium was added to a 25-30% confluent 10cm plate of the primate specific retroviral packaging cell line PG13.

35 After overnight incubation, the medium was changed and the cells were grown to between 36 and 48 h post infection. At this time, the cells were trypsinized and reseeded in 10cm plates at different plating densities in DME, 10% FBS, 500  $\mu$ g/ml (active concentration) of G418. This selective medium was changed with 2 day intervals until colonies of 2-3 mm dia. were visible. These colonies were isolated by scraping off and aspirating in <10 ml with a micropipet.

The isolated cells were trypsinized in 50 ml trypsin solution, which was added to 4 ml of DME, 10% FBS in the well of a six-well plate. When confluent, the cells were again trypsinized and reseeded into one 10 cm dish and one square dish with three microscope slides for immunostaining.

5 Five PG13 clones were stained for surface expression of the sFv. Clones PG13-NC3#1, #5, #7, #8, #9 were stained as described above but using CD28-hIg-biotin at 10 µg/ml, 60 min, RT, followed by streptavidin-FITC at 5 µg/ml, 30 min, RT. All clones were positive, but clones PG13-NC3#5, #7, #8 showed best expression. All clones were frozen in LN2.

10 Infection and subcloning of human Hela, H3347 and 2987 carcinoma cells was done using virus-containing medium from Pg13-NC3#5 cells as described above for viral infection of PG13 cells and selection was in DME, 10% FBS, 500 µg/ml G418. Clones were tested by immunostaining with goat anti-human Ig-FITC and/or CD28hIg-biotin. Hela clones NC3-2, -6, -8, -13 and -15 were frozen. H3347 and 2987 clones 15 were tested before subcloning and were positive for surface expression of 2E12-hIgG1, but subclones were not tested. Twelve clones of each line were frozen for future testing.

20 Hela cells infected with retroviral constructs NC3-2 (—), 58-32 (---), B7-1 (-----), or non-infected cells (....) (Figure 1). Figure 1 shows that Hela cells infected with the 58-32 or NC3-2 retroviruses express the 2E12 sFv on the cell surface as detected either by binding to CD28 or by binding to anti-human Ig. The Hela cells infected with the B7-1 retrovirus express high levels of B7-1.

25 The GPI anchor from CD58 is cleaved by phospholipase C in the NIH3T3 retrovirus packaging cell line, but is resistant to phospholipase C digestion in the 58-32 Hela cell line (Figure 2). NIH3T3 virus packaging cells (A) or Hela cells (B) infected with retroviral constructs containing the 2E12 sFv-Ig fusion protein linked to a CD58 GPI anchored tail. Surface expression of the 2E12 sFv was analyzed by flow cytometry prior to (—) or following (—) PI-PLC digestion.

30 Figure 3 is a bar graph showing stimulation of proliferation of T cell blasts with Hela cells expressing anti-CD28 sFv or B7-1 on the cell surface. Rested day 7 PHA T cell blasts were cocultured for 2 days with irradiated Hela cells or Hela expressing B7-1 or the CD28 sFv at a 1:10 ratio of stimulator cells to T cells, and proliferation was measured by uptake of <sup>3</sup>H-thymidine for the last 6 hours. Mean proliferation was determined from quadruplicate cultures, and the standard errors were less than 10% of the mean at each point. CTLA4-Ig (2 µg/ml) was added to the cultures as indicated.

35 This shows that the signal to the T cells is specific for the CD28 receptor, and that the activity is not due to a ligand that binds to CTLA-4, such as B7-1 or B7-2.

In Figure 4, T cells were present at a constant  $5 \times 10^4$  cells per well, while the irradiated Hela cell lines were titered as indicated. Proliferation was measured by incorporation of  $^3$ H-thymidine in quadruplicate cultures of a 96 well microtiter plate, and standard errors did not exceed 10% of the mean at any point.

5 This shows that the Hela NC3-2 and Hela 58-32 cells expressing the 2E12 sFv on the surface were comparable to the Hela B7-1 cells for their ability to induce proliferation.

In Figure 5, cells were pelleted and rapidly lysed in 1% NP40-containing lysis buffer and nuclei were removed by centrifugation. The vav protooncogene was  
10 immunoprecipitated from the lysates using 4  $\mu$ g of polyclonal anti-vav followed by Protein A-Sepharose (Koch et al. (1991) *Science* 252:668). The immunoprecipitates were extracted in SDS sample buffer and electrophoresed on 10% polyacrylamide gels. The gel was then transferred to a PVDF filter and blotted with rabbit anti-phosphotyrosine Ab followed by detection with  $^{125}$ I-Protein A and autoradiography.  
15 The PVDF filter was then stripped with 2 washes using pH 2.2 glycine-HCl at 70°C for one hour each. The filter was then blotted again with rabbit anti-vav to determine the amount of vav present in each lane.

Figure 5 shows that the Hela cells expressing the cell surface 2E12 sFv are equivalent to the Hela cells expressing B7-1 for their ability to rapidly activate the  
20 tyrosine kinases that phosphorylate the vav protooncogene.

## EXAMPLE 2

25 **Construction of the retroviral vector pLNC-2e12-hIgG1-hCD58GPI:** The sequence of a cDNA for the human GPI (glyco-phosphoinositol) linked form of CD58 (LFA-3) has been reported (Seed, B. 1987, *Nature*, 329:840-842). From this sequence, bases 631 through 739 were isolated by PCR amplification (Figure 12F). The fragment encodes aa 207 through 237, i.e. the C-terminal signal sequence and 9 aa of the extracellular domain, including the serine attachment site for the GPI anchor.

30 Two oligonucleotides were synthesized, covering the bases 631-739 with a 17 base overlap. These were: SCD58GPI, 5'-CTGCATCCTGGAT CCA AGC AGC GGT CAT TCA AGA CAC AGA TAT GCA CTT ATA CCC ATA CCA **TTA GCA GTA ATT AC-3'** and ACD58GPI, 5'-CAGCGTTGCTCGAGATTGTCTTCTCAA TTA AAG AAC ATT CAT ATA CAG CAC AAT ACA TGT TGT AAT TAC TGC TAA  
35 **TGG-3'.** The bases in bold face indicate the overlapping sequence.

In addition, two PCR primers were used: SCD58BAM, comprising the first 36 bases of SCD58GPI and ACD58XHO, comprising the first 43 bases of ACD58GPI.

The CD58 GPI anchor was amplified using 10nM each of SCD58GPI and ACD58GPI and 1 mM each of SCD58BAM and ACD58XHO primers with 4 initial cycles at 94 C, 30 sec., 45 C, 30 sec., 72 C, 2 min. and 30 subsequent cycles at 94 C., 30 min., 68 C, 30 sec., 72 C, 2 min. The 137 base pair product was purified and restricted with BamH-I and Xho-I and the resulting 117 bp product was purified over a QIA Quick cartridge and ligated at a 2:1 molar ratio into the BamH-I and Xho-I cleaved pSFVDNA-1-2V7 vector (see above). Briefly, the 50 ml ligation reaction contained 0.07 pmole of the 2V7 vector and 0.15 pmole of the CD58GPI fragment.

Of 18 colonies screened, 16 contained the correct BamH-I/Xho-I fragment. Clones pSFVDNA-1-2e12-hIgG1(Fc-)-CD58GPI#58-2, #58-3, #58-6 were tested (06/21/95) for expression in Cos cells, by membrane staining with goat anti-human IgG-FITC, CD28-hIgG-biotin and mAb HP6058 (mouse anti-human IgG1, 2, 3 Fc region). The Hind-III/Cla-I insert in pSFVDNA-1 clones 58-2, 58-3 and 58-6 were separately cloned into Hind-III/Cla-I cleaved pLNCX vector and one correct clone from each ligation, pLNC2e12-hIgG1-(Fc-)-CD58GPI #58-21, #58-32, #58-64, was further tested by transient expression in Cos cells. All three clones were positive for membrane staining with goat anti human IgG-FITC and CD28-hIgG-biotin. Cos cells simultaneously transfected with a pLNCX clone of the human B7-1 (CD80, see below) was used as a control. PG13 packaging cells were generated for the three retroviral clones pLNC-2e12-hIgG (Fc-) CD58GPI #58-21, #58-32, #58-64 as described above. Bulk G418 selected PG13 cells were tested and found positive for membrane staining with goat anti-human IgG-FITC and CD28-hIgG-biotin. Pg13 clones were isolated from #58-32 and #58-64 cells, and were retested for expression as above.

**Construction of the pLNC-hB7-1 retroviral vector:** A retroviral vector expressing the normal human B7-1 cDNA was constructed as a control for comparative analyses of the functions of the membrane anchored forms of the 2E12 sFv (Figure 9). This vector is isogenic with the constructs described above, except with regard to the CD28 ligand moiety. A clone of the human B7-1 cDNA in the vector pCDNA-1 was configured for cloning into the Hind-III/Cla-I cleaved pLNCX vector by PCR.

The primers used were SHB71ATG: 5'GTGAATTCCAAGCTTCCACC ATG GGC CAC ACA CGG AGG CAG-3' and AHB71TM, described above. Amplification conditions were 30 cycles at 94 C, 30 sec., 68 C, 1 min., 72 C, 2 min. The 908 bp PCR

product was cleaved with Hind-III and Cla-I and the resulting 888 bp cleavage product was gel-purified and ligated into the pLNCX vector. Three clones, pLNC-hB7-1#1, #2, #3 were tested for expression by transfection into Cos cells. All three were positive by staining with anti-B7-1 mAb (B&D, BB-1) followed by goat anti-mouseIg-FITC or with 5 CD28Ig-biotin, followed by streptavidin-FITC.

PG13 packaging cells were generated as described above. These were immunostained after G418 selection as above and were found positive, and clones were isolated and tested. Clone PG13-B7-1#12 (B7-112) showed the strongest surface fluorescence. Several clones were frozen.

Table 1

**T-cell growth stimulation by HeLa cells expressing  
hB7-1 or membrane-bound 2E12 sFv.**

5

<u>Presenting cells:</u>	<u>- blasts</u>	<u>+ blasts</u>
None		16701 (1%)
CHO B7-1	<1000	128402 (3%)
HeLa	<1000	2501 (2%)
HeLa B7-1 <sup>2</sup>	<1000	67246 (1%)
HeLa NC3-2 <sup>3</sup>	<1000	120729 (6%)
HeLa NC3-6 <sup>3</sup>	<1000	118310 (3%)
HeLa 58-3 <sup>4</sup>	<1000	144502 (2%)
HeLa 58-6 <sup>4</sup>	<1000	61422 (<1%)
 <b>a-CD28 mAb:</b>		
9.3 mAb		58782 (<1%)
9.3 mAb + 187.1 Ab		98303 (3%)

<sup>1</sup> Average of three cultures of  $4 \times 10^4$  7-day PHA blasts +  $10^4$  irradiated target cells incubated 3 days followed by 6 h. pulse w.  $^3$ H-Thymidine.

10   <sup>2</sup> HeLa cells inf. with pLNC-hB7-1 virus.

<sup>3</sup> HeLa cells inf. with pLNC-2e12-Ig-B7-1(Tm) virus.

<sup>4</sup> HeLa cells inf. with pLNC-2e12-Ig-CD58GPI virus.

**EXAMPLE 3****MATERIALS AND METHODS**

**PCR Amplification of Variable Region Genes and Construction of Expression Cassettes:** Total cellular RNA from  $5 \times 10^7$  hybridoma cells was isolated 5 using rapid lysis in NP-40 or a modification of the single step acid-guanidinium thiocyanate protocol (Chomczynski P., et al. Single step method of RNA isolation by acid guanidinium thiocyanate phenol-chloroform extraction. *Anal Biochem* 1987: 162: 156-159.). RNA was reverse transcribed with AMV reverse transcriptase (Life Sciences) and either random primers or antisense oligonucleotides that annealed to 10 specific mouse kappa light chain or heavy chain constant region sequence approximately 100 bases downstream of the J-C junction. Typically, 10  $\mu$ g RNA and 1  $\mu$ g primer was used to generate cDNA.

The first strand was then poly-G tailed using terminal transferase (Stratagene), dGTP, and an antisense nested primer that annealed approximately 50 bases from the 15 murine kappa light chain or heavy chain constant region J-C junction. The tailed cDNA was amplified by PCR as described previously (Gilliand LK, et al. Rapid and reliable cloning of antibody variable regions and generation of recombinant single chain antibody fragments. *Tissue Antigens* 1996: 47: 1-20).

Anti-L6 mAb is specific for a cloned tumor antigen (Marken JS, et al. 20 Cloning and expression of the tumor associated antigen L6. *Proc Natl Acad Sci USA* 1992: 89: 3503-3507.) that is expressed at high levels on certain human tumors (Fell HP, et al. Chimeric L6 anti-tumor antibody. Genomic construction, expression, and characterization of the antigen binding site. *J Biol Chem* 1992: 267: 15552-15558. The sFv for L6 was cloned and expressed as described previously (Hayden MS, et al. 25 Single-chain mono-and bispecific antibody derivatives with novel biological properties and anti-tumor activity from a COS cell transient expression system. *Ther Immunol* 1994: 1: 3-15.; Fell HP, et al. Chimeric L6 anti-tumor antibody. Genomic construction, expression, and characterization of the antigen binding site. *J Biol Chem* 1992: 267: 15552-15558). 30 Secondary PCR was used to add restriction sites for subcloning into the appropriate locations. The bispecific  $\alpha$ CD28- $\alpha$ L6 (9.3/L6) sFvIg fusion cassette was constructed by cutting the 9.3 sFvIg at BcII and inserting an L6 sFv as a BcII-BcII fragment directly adjacent to the 3' end of 9.3. The 2e12 mAb is a second mouse anti-human CD28 specific molecule, and the sFv was cloned and expressed as described

elsewhere (Gilliand LK, et al. Rapid and reliable cloning of antibody variable regions and generation of recombinant single chain antibody fragments. *Tissue Antigens* 1996; 47: 1-20).

**Plasmid Vectors for Expression of Soluble and Membrane Bound sFv: A**

5 modified version of the mammalian expression vector pCDM8 has been described previously (Hayden MS, et al. Single-chain mono-and bispecific antibody derivatives with novel biological properties and anti-tumor activity from a COS cell transient expression system. *Ther Immunol* 1994; 1: 3-15.). In addition to this vector used for transient expression of sFv fusion proteins, a second vector has been generated for use

10 in generating stable cell lines expressing these fusion proteins. Briefly, the sFvIg cassettes were subcloned as HindIII-XbaI fragments into the polylinker region adjacent to the CMV promoter in a modified version of pD16, a derivative of pCDNA3 (Invitrogen). The vector contains a DHFR gene with an attenuated promotor and an AscI linearization site in a second polylinker region outside of the

15 expression cassette and DHFR regions. Several restriction sites present in the original pD16 plasmid were removed by PCR mutagenesis to simplify subcloning of -Ig fusion proteins into the pD18 vector.

A modified version of the retroviral expression vector pLNCX was generated as described previously. This vector was then used to construct a 2e12-Ig-B7-1

20 transmembrane and cytoplasmic tail (TM+CT) fusion cassette for cell surface expression of sFv molecules. The 9.3 sFv region was cut with HindIII and BcII and substituted for the 2e12 HindIII-Bg1II sFv fragment, creating a second CD28 sFv fusion cassette attached to the hulgG1 and CD80 transmembrane and cytoplasmic tail.

**Generation of Tumor Lines Expressing Membrane Bound sFV and**

25 **CD80:** Gene fusion constructs of the sFv or CD80Igs in pLNCX were transfected into PE501 ecotropic packaging cells by CaPO<sub>4</sub> precipitation. After 16 hours of incubation, the medium was replaced (DMEM/10% FBS), and transiently produced retrovirus was recovered in the culture medium after a further 24 hour incubation. The virus containing medium (10 ml) was filtered through a 0.2 µm Acrodisc syringe

30 filter (Gelman). Hexadimethrine bromide was added to a final concentration of 8 µg/ml and the medium was added to a 25-30% confluent 10 cm plate of the primate specific retroviral packaging cell line PG13.

After overnight incubation, the medium was changed and the cells were grown for 24 - 36 hours post infection. At this time, the cells were treated in Versene (PBS) and reseeded 0.2g/L EDNA-4Na ph 7.0 in 10 cm plates at different plating densities in

DMEM/10% FBS. After 24 hours, media was changed to DMEM/10% FBS/500 µg/ml G418.

The media was changed in 2-day intervals until colonies of 2-3 mm diameter were visible. The colonies were isolated by scraping and aspirating in <10 µl using a 5 micropipeter. The isolated colonies were trypsinized in 100 µl versene solution, which was then added to 4 ml DMEM/10% FBS/500 µg/ml G418 in a single well of a 6 well cluster plate.

When confluent, the cells were again Versene and reseeded into one 10 cm dish. Individual clones of cells were isolated and stained for 2e12 or 9.3 sFvIg 10 surface expression. Two clones for 9.3 (designated #4-1 and #6-4), and one each for 2e12 (designated 3-2/3c) and CD80 (designated B7-112) were selected for further use because of higher expression levels of the molecules on the cell surface.

H3347 tumor cells expressing high levels of the L6 antigen were transfected with retroviral vectors containing membrane bound versions of the 9.3 (designated 9.3 15 4-1 b1 and 9.3 6-4 a4) and 2e12 (designated 3-2/3c) anti-CD28 sFvIg. Similar stable lines were generated which expressed human CD80 (designated B7-112).

Infection and subcloning of H3347 tumor cells was done using virus containing media from these PG13 clones. H3347 clones were all positive for 9.3 sFvIg surface expression by staining with biotinylated-CD28Ig (B-CD28Ig) followed 20 by streptavidin-phycoerythrin (SA-PE); or FITC-anti human IgG.

**Cell Culture, Transfection, and Purification of Soluble sFvIg Molecules:**  
The hybridoma producing murine anti-human CD28 (9.3) was used. Additionally, the anti-L6 human tumor specific hybridoma and 2e12 anti-hCD28 hybridoma were used.

COS cells were transfected with sFvIg expression plasmids isolated from 3-6 25 clones of MC1061/p3 transformants as previously described (Hayden MS, et al. Single-chain mono-and bispecific antibody derivatives with novel biological properties and anti-tumor activity from a COS cell transient expression system. Ther Immunol 1994: 1: 3-15; Gilliland LK, et al. Rapid and reliable cloning of antibody variable regions and generation of recombinant single chain antibody fragments. 30 Tissue Antigens 1996: 47: 1-20). Three days following transfection, culture supernatants were collected and tested for the presence of sFv-Ig fusion protein, and for specific binding activity of the protein.

Positive clones from this initial screening were then selected and large scale transfections performed. Usually 500-1000 ml of serum free supernatant was

collected over a nine day culture period. Protein was isolated and purified from culture supernatants as described previously (Hayden MS, et al. Single-chain mono- and bispecific antibody derivatives with novel biological properties and anti-tumor activity from a COS cell transient expression system. *Ther Immunol* 1994: 1: 3-15; 5 Gilliland LK, et al. Rapid and reliable cloning of antibody variable regions and generation of recombinant single-chain antibody fragments. *Tissue Antigens* 1996: 47: 1-20).

Stable CHO lines were generated by high copy electroporation of CHO DG44 cells (Barsoum J. Introduction of stable high-copy-number DNA into Chinese 10 hamster ovary cells by electroporation. *DNA Cell Biol* 1990: 9: 293-300; Urlaub G, et al. Effect of gamma rays at the dihydrofolate reductase locus: deletions and inversions. *Somat Cell Mol Genet* 1986: 12: 555-566.) with linearized pD18 expression plasmid containing the sFvIg expression cassettes. Approximately 250 µg plasmid DNA was digested with Ascl, phenol/chloroform extracted, and 15 coprecipitated with 200 µg sheared herring sperm DNA as carrier.

Transfections were performed by mixing  $1 \times 10^7$  CHO DG44 cells (Urlaub G, et al. Effect of gamma rays at the dihydrofolate reductase locus: deletions and inversions. *Somat Cell Mol Genet* 1986: 12: 555-566) with DNA in 0.8 ml PFCHO (JRH Biosciences) containing 4 µg/ml hypoxanthine, 0.72 µg/ml thymidine, 4mM 20 glutamine, and 0.5 µg/ml recombinulin insulin in an electroporation cuvette (Biorad) and electroporating at 300 volts, 960 µF. Cells were transferred to T25 flasks and incubated in 10 ml non-selective media for 1-2 days prior to plating in selective media containing 100 nM methotrexate.

Transfected clones were ready to screen by ELISA within 2-3 weeks of 25 plating. ELISAs were performed as described previously (Gilliand LK, et al. Rapid and reliable cloning of antibody variable regions and generation of recombinant single chain antibody fragments. *Tissue Antigens* 1996: 47: 1-20), with serial dilutions from a 1:100 starting solution of culture supernatant. Clones expressing higher levels of the fusion proteins were amplified for 7-12 days in 6% CO<sub>2</sub> gassed spinner flasks 30 containing selective media. Cultures were filtered through Gelman suporcap-50 or 100 0.2 µm filters into roller bottles using a Cole-Parmer Masterflex pump and pump drive prior to protein A purification.

**SDS PAGE and Western Blotting:** SDS PAGE and Western Blotting were performed as described previously (Hayden MS, et al. Single-chain mono- and 35 bispecific antibody derivatives with novel biological properties and anti-tumor

activity from a COS cell transient expression system. *Ther Immunol* 1994; 1: 3-15; Gilliland LK, et al. Rapid and reliable cloning of antibody variable regions and generation of recombinant single chain antibody fragments. *Tissue Antigens* 1996; 47: 1-20).

5 **Immunostaining and FACS analysis:** Binding of antibodies and fusion proteins to Jurkat T cells, CD28CHO cells, L6 positive tumor cells, transfected tumor cells, or purified T lymphocytes was analyzed by indirect immunofluorescence. Single cell suspensions were obtained by treating monolayer cultures with a solution of EDTA (0.2 g/L) dissolved in PBS.

10 Cells were incubated with antibodies or fusion proteins at the indicated concentrations in staining media (RPMI 1640 + 5% FBS + 0.1% sodium azide) for 1 hour on ice. Cells were washed and bound proteins detected with goat anti-mouse IgG or anti-human IgG conjugated to FITC (Biosource International) for 40 minutes on ice.

15 Some assays were performed by a three step incubation involving the anti-CD28 fusion protein first, followed by biotinylated CD28Ig or  $\alpha$ L6Ig, and then PE conjugated streptavidin (PE-SA). Other assays with sFv transfected tumor cells involved incubation in biotinylated CD28Ig, L6, or  $\alpha$ CD80, followed by PE-SA. Cells were washed with ice-cold staining media and fixed in PBS containing 0.2%  
20 paraformaldehyde prior to fluorescence analysis using a FACSCAN cell sorter (Becton Dickinson and Co.). Usually 10,000 cells were analyzed per sample.

25 **Proliferation Assays:** Lymphocytes were isolated from peripheral blood of healthy human volunteers using Lymphoprep Separation Media (Organon Teknika). PHA activated T cell blasts were prepared by culturing PBL with 1  $\mu$ g/ml PHA (Wellcome) for 6 days, and resting 1 day in media lacking PHA. L6-positive tumor cells (H2981, H3347, and CD28- or CD80- transfected H3347 cells) were exposed to 5,000 rads prior to use in proliferation assays.

30 Lymphocytes were cultured in round-bottom 96-well tissue culture plates (Costar) at  $5 \times 10^4$  cells/well in RPMI 1640 medium containing 10% FBS in a final volume of 0.2 ml. Fusion proteins were tested as purified protein at the concentrations indicated. Fusion proteins were either added in solution to PBL, or where indicated, were prebound to the tumor cells with soluble protein removed by washing before incubating with PBL. Proliferation was measured in triplicate

samples by uptake of [<sup>3</sup>H] thymidine at 1  $\mu$ Ci/ml added during the last 6 hours of a three day culture.

**Cloning and sequencing 9.3 variable region genes:** The 9.3 hybridoma produces antibody specific for human CD28 expressed on T cells (Jung G, et al.

5 Induction of cytotoxicity in resting human T lymphocytes bond to tumor cells by antibody heteroconjugates. Proc Natl Acad Sci USA 1987: 84: 4611-4615). The isotype of the antibody is mouse IgG2a with k light chain, so the primers used for the first strand synthesis step were either random primers or the specific primers for heavy and light chain constant regions mIgG2a-1 (VH) with the sequence

10 5'CAGGTCAAGGTCACTGGCTCAGG-3', and mIgck-1 (VL) with the sequence 5'CTTCCACTTGACATTGATGTCTTG-3' (Ollo R, et al. Comparison of mouse immunoglobulin gamma2a and gamma2b chain genes suggests that exons can be exchanged between genes in a multigenic family. Proc Natl Acad Sci USA 1981: 78: 2442-2446). The VH and VL cDNA were poly-G tailed and were then amplified by

15 PCR using the ANCTAIL 5' primer 5'CGTCGATGAGCTCTA-GAATTCCGCAT GTGCAAGTCCGATGGTCCC-CCCCCCCCCCC-3' (Gilliand LK, et al. Rapid and reliable cloning of antibody variable regions and generation of recombinant single chain antibody fragments. *Tissue Antigens* 1996: 47: 1-20) and the nested 3' primers HBS-mG2a (V<sub>H</sub>) 5'CGTCATGTCGACGGATCCC-

20 AAGCTTGAGCCAGTTGTATCTCACACACAG-3' (24) and HBS-mck (V<sub>L</sub>) 5'- CGTCATGTCTGACGGATCCA AGCTTCAAGAAGCACACGA-CTGAGGCAC-3' (Altenburger W, et al. DNA sequence of the constant gene region of the mouse immunoglobulin kappa chain. *Nucleic Acids Res* 1981: 9: 971-981).

A single DNA band of approximately 500 bp was observed after agarose gel electrophoresis of aliquots from each PCR reaction, representing leader, V gene, and about 50 bases encoding the constant region. PCR products were then restriction digested with Hind III and XbaI and purified fragments subcloned into pUC19, amplified in DH5 $\alpha$ , and plasmid DNA prepared to screen for inserts. Subclones containing 500 bp inserts were then subjected to DNA sequence analysis and 3-4

25 clones used to determine consensus sequence for the variable regions.

Subclones with correct sequence were then used as templates in PCR reactions that attached the appropriate sequences to fragments for assembly of an sFv. Rather than performing SEWING PCR, a Bam HI restriction site was introduced at the 3' end of the (gly<sub>4</sub>ser)<sub>3</sub> linker, and the entire linker sequence was attached to the 3' end of V<sub>L</sub>

30 by PCR, using a 78-mer oligonucleotide with the following sequence:

5'CTGGGCCTGGATCCACCGCCGCCT  
GAACCGCCACCTCCAGAACCGCCACCACCGAAGCCCCTTTATTCAG

CTT3'. The native leader for the 9.3 V<sub>L</sub> was used for assembly of the sFv and a HindIII site was attached by PCR using the following 36-mer oligonucleotide as

5 primer: 5'GGACTGCTGAAGCTTATG GAGTCAGACACACTCCTG3'.

PCR products were then digested with HindIII and BamHI, and subcloned into pUC19. BamHI and BclI sites were attached to the 5' and 3' ends of the V<sub>H</sub> domain using the 36-mer sense primer 5'CTGGACTGGATCCCTGGCTCAGGT GCAGCTGAAG-3' and the 39-mer antisense primer

10 5'GGTGGAGGTTGATCAGAGG AGACGGTGACTGAGGTTCT3'.

The PCR product from the V<sub>H</sub> domain was subcloned into a pUC19 vector containing L6 sFvIg which had been digested with BamHI and BclI. The 9.3 V<sub>H</sub>Ig vector was then digested with HindIII and BamHI and ligated to the HindIII+BamHI (V<sub>L</sub>-linker) cassette. DNA from these transformants was then digested with HindIII 15 and XbaI to screen for a full length sFvIg fragment. The HindIII-XbaI fragments with the appropriate size for a full length sFvIg were transferred to the expression vector pCDM8 and DNA prepared for transfection of COS cells. The nucleotide and deduced amino acid sequence for the 9.3 sFv, including the V<sub>L</sub>-leader, V<sub>L</sub>, linker, and V<sub>H</sub>, is shown in Figure 13.

20 **Production, Expression, and Screening of 9.3 sFvIg and 9.3/L6 sFvIg**

**Fusion Proteins:** COS cell supernatants were screened for the presence of 9.3 sFvIg fusion protein by IgG sandwich ELISA and by immunoprecipitation with protein A, SDS-PAGE, and Western blotting to visualize precipitated proteins. Using these two screening assays, two out of three clones were shown to express protein reactive with 25 anti-human Ig reagents. Upon sequencing, the clone that was negative for protein expression was found to contain a Tyr to Cys mutation in the CDR3 region of the heavy chain variable region, indicating that a PCR induced mutation accounted for the lack of expression.

The 9.3/L6 bispecific fusion cassette was created by fusing the αCD28 and 30 αL6 V<sub>L</sub>V<sub>H</sub> cassettes directly to one another without any intervening linker sequence following directly after the JH4 region of 9.3. The bispecific expression cassette was inserted into the pCDM8 expression vector at the same location as the monospecific sFvIg constructs. Each of the L6 sFvIg, 9.3 sFvIg, and 9.3/L6 sFvIg gene fusions were transfected into COS cells and crude culture supernatants assayed for the 35 presence of protein as described above.

Figure 14 shows the results of Western Blot analysis on the 9.3 sFvIg, L6 sFvIg, mock transfected, and 9.3/L6 sFvIg transfection supernatants. COS cell supernatants were immunoprecipitated with *Staphylococcus* protein A, washed, and subjected to SDS-PAGE. Gels were blotted to nitrocellulose and incubated with 5 alkaline-phosphatase conjugated goat anti-human IgG to visualize proteins. SFvIg proteins for L6 (Lane 4) and 9.3 (Lane 2) migrate at  $M_r$  55,000, the approximate size expected for these fusion proteins. Supernatants from mock transfected cells (Lane 1) show no protein, while supernatants from the 9.3/L6 sFvIg bispecific (Lane 3) migrate at  $M_r$  83,000, the approximate size expected for the bispecific protein.

10 **Binding activity of the 9.3 and 9.3/L6 sFvIg Fusion Protein:** The binding of each sFvIg fusion protein was assayed by immunostaining and FACS analysis. The bispecific 9.3/L6 sFvIg, 9.3 sFvIg and L6 sFvIg molecules at 1  $\mu$ g/ml were 15 incubated with H2981 L6 positive tumor cells to assay binding to the L6 molecule as shown in Figure 15A. The 9.3 sFvIg and the negative control including only second step were identical and are shown in the Figure as control.

9.3 sFvIg and 9.3/L6 sFvIg were incubated with CD28 CHO cells and binding was detected using FITC anti-human IgG as shown in Figure 15B. Purified CD28<sup>+</sup> T cells were incubated with (1) FITC labeled anti-L6 idiotype 13B, (2) the bispecific 9.3/L6 sFvIg followed by FITC 13B, or (3) preblocked with 9.3 sFvIg, then bound to 20 9.3/L6 sFvIg and FITC 13B as shown in panel 15C. The results of these assays demonstrate functional binding activity for both the 9.3 sFvIg and the 9.3/L6 sFvIg. Staining with either the mono- or bispecific fusion proteins plus second step was 25 similar to staining with native 9.3 antibody. Staining for the L6 portion of the bispecific fusion protein was reduced when compared to native L6 antibody or the L6 sFvIg monospecific construct so that the binding observed at equivalent concentrations was comparable to that of the L6 antibody at half the concentration (1  $\mu$ g/ml looks like 0.5  $\mu$ g/ml native antibody), indicating that the binding activity of the second sFv (L6) in this particular fusion protein was less than that of the first sFv (9.3). The reduced level of binding may be due to the absence of a linker region 30 between the sFvs in this bispecific molecule.

**Comparison of Costimulation by soluble 9.3 sFvIg and soluble CD80Ig:**  
To determine if soluble 9.3 sFvIg was equivalent to CD80Ig at providing 35 costimulatory signals to T cells, 7-day resting PHA blasts were co-cultured for three days with equal concentrations of the sFvIg or CD80Ig molecule with and without anti-human IgG or protein A as crosslinking reagents. [<sup>3</sup>H]-thymidine incorporation

was measured during the last 6 hours of the assay. 9.3 sFvIg and CD80Ig molecules were present in soluble form at 0.02  $\mu$ g/ml, 0.1  $\mu$ g/ml, 0.5  $\mu$ g/ml, or 1.0  $\mu$ g/ml.

Stimuli were incubated with no crosslinking, anti-human IgG at 4:1 (protein: IgG), or protein A at 4:1 (protein: protein A). The results of this experiment are shown in

5 Figure 19. The CD80Ig molecule alone generates little or no costimulatory signal without crosslinking, although crosslinking enhances the signal somewhat at higher concentrations. In contrast, the 9.3 sFvIg is able to generate stronger costimulatory signals than CD80Ig under identical conditions. Crosslinking greatly enhances costimulation generated by the 9.3 sFvIg. Although the magnitude of costimulation fluctuated from experiment to experiment and from donor to donor, more significant 10 responses were obtained using 9.3 sFvIg than the CD80Ig at every concentration tested and for every experimental treatment. Similarly, crosslinking enhanced costimulation by 9.3 sFvIg more significantly than by CD80Ig. In some experiments, CD80Ig was found to suppress costimulation rather than enhancing it.

15 **Titration of 9.3/L6 sFvIg mitogenicity in resting PHA blasts:** The ability of the bispecific 9.3/L6 sFvIg fusion protein to provide costimulatory signals to T cells and the levels of protein required to observe costimulatory effects were examined. Rested PHA blasts were incubated with dilutions of bispecific 9.3/L6 sFvIg fusion protein at concentrations of 0.5  $\mu$ g/ml, 0.1  $\mu$ g/ml, and 0.02  $\mu$ g/ml. As 20 shown in Figure 16, higher concentrations of bispecific protein (0.5  $\mu$ g/ml) gave proliferation without addition of other reagents, although the mitogenic effects of the molecule were augmented by the presence of crosslinking reagents or L6 positive tumor cells. However, at lower concentrations of bispecific 9.3/L6 sFvIg, crosslinking of the molecule by protein A, anti-L6 idiotype 13B, or H3347 tumor cells 25 was required to generate a significant proliferative response.

In Figure 16, seven day PHA blasts ( $5 \times 10^4$  cells/well) were incubated with dilutions of bispecific 9.3/L6 sFvIg fusion protein at concentrations of 0.5, 0.1 and 0.02  $\mu$ g/ml. At each dilution, the sFvIg and blasts were coincubated with 0.5  $\mu$ g/ml protein A,  $\alpha$ -L6 idiotype 13B (2  $\mu$ g/ml), H3347 tumor cells, or no other reagent.

30 H3347 cells were irradiated at 5,000 rads, preincubated with sFvIg, washed, and incubated at a 1:5 E:T cell ratio in the indicated samples. All data are obtained from triplicate samples for each treatment, and SEM are <6%.

These results show that at high concentrations, greater than 0.1  $\mu$ g/ml, bispecific 9.3/L6 sFvIg alone can induce proliferation of T cell blasts. This activity is 35 not dependent on binding of the L6 variable region of the bispecific, but occurs by

CD28 mediated activation signals only, a pattern of costimulation that may be undesirable for tumor targeting. At these high concentrations, a significant increase in proliferative responses results from coincubation with L6 positive tumor cells or crosslinking reagents. However, 0.02 µg/ml of fusion protein requires the presence of 5 tumor cells or crosslinking to produce significant proliferation, so we chose to explore costimulation under these conditions in more detail.

**Comparison of the abilities of 9.3/L6 sFvIg and 9.3 sFvIg molecules to stimulate T cell proliferation in the presence of tumor cells or crosslinking reagents:** The conditions necessary to produce T cell proliferation dependent upon 10 both the CD28 and L6 binding activities of the bispecific protein were determined. Figure 17 shows the results of an assay where resting PHA blasts were incubated with low concentrations (20 ng/ml) of bispecific 9.3/L6, 9.3 sFvIg fusion protein, or media, where crosslinking of surface bound CD28 and costimulation does not occur in the presence of these stimuli alone.

15 In Figure 17, seven day resting PHA blasts ( $5 \times 10^4$  cells/well) were incubated with either monospecific or bispecific fusion protein at 20 ng/ml, a concentration where CD28 receptor binding alone fails to result in costimulation. The molecules were crosslinked through their Fc domain with protein A at 0.5 µg/ml, through the L6 epitope with L6 anti-idiotype 13B at 2 µg/ml, or by actively binding L6 antigen on the 20 surface of H3347 tumor cells. H3347 tumor cells were irradiated, preincubated with sFvIg, washed, and incubated at a 1:5 E:T cell ratio in the indicated wells. The results demonstrate that the bispecific molecule but not the 9.3 sFvIg can be crosslinked by coincubation with H3347 tumor cells and PHA blasts, generating a powerful mitogenic signal for T cell proliferation.

25 Monospecific 9.3 sFvIg was mitogenic for T cell proliferation when crosslinked using protein A but failed to stimulate proliferation when incubated with media, mAb 13B, or irradiated H3347 tumor cells. Only bispecific 9.3/L6 sFvIg was mitogenic in the presence of 13B or tumor cell crosslinking, demonstrating that the costimulatory activity of the 9.3/L6 sFvIg at low concentrations was dependent either 30 on engagement of both sFvs or on some less tumor specific method of achieving crosslinking. Because crosslinking of surface bound CD28 is required for costimulatory signals, engaging both of the binding sites of the bispecific molecule must be sufficient to crosslink the CD28 receptor and trigger T cell activation.

35 For successful tumor targeting, stimulation should be limited to those instances where the costimulatory molecule has actually bound to its tumor antigen

target and not occur from an antigen-independent engagement of the CD28 receptor alone. The data above demonstrate that at low concentrations, costimulation requires crosslinking of the bispecific molecule to stimulate proliferation, and that binding to both the T cell surface and tumor antigen is sufficient to crosslink the CD28 receptor 5 on the T cell surface.

**Generation of H3347 experimental tumor lines expressing membrane bound CD80 and 9.3 sFv:** The costimulatory activity generated by bispecific sFv coated tumor cells was compared to that generated by tumor cell surface expression of sFv. H3347 tumor cells were transfected with the pLNCX retroviral expression 10 vector containing CD80-anchored 2e12 sFvIg, CD80-anchored 9.3 sFvIg, or the native CD80 molecule. Transfectants were assessed for cell surface expression of the sFvIg or CD80 molecule by indirect immunofluorescence using FITC anti-human IgG (all samples), biotinylated CD28Ig (B-CD28Ig) and PE-streptavidin (9.3 transfectants and 9.3/L6 sFvIg), B-L6 and PE-streptavidin (H3347 cells) or B- $\alpha$ CD80Ig plus PE-streptavidin (for CD80 transfectants) followed by flow cytometry analysis.

The results shown in Figures 18a-g demonstrate the specificity of staining using these reagents for both transfected tumors and 9.3/L6 sFvIg bound tumors. Those transfectants expressing the CD28 sFvs gave positive signals for binding to both CD28Ig and FITC anti-human IgG. 20 The CD80 expressing tumors were negative for binding FITC anti-human IgG and B-CD28Ig/PE-streptavidin yet positive for binding to the B- $\alpha$ CD80/PE-streptavidin combination. The CD28Ig-B7 binding interaction is apparently too weak to detect with the B-CD28Ig, so that only B7 specific antibody was positive for binding. The expression level of sFv or B7 varies from clone to clone, with the 25 lowest level expressed by 9.3 clone 6-4, followed by 9.3 clone 4-1, B7-112, and 2e12 clone 3-2/3c. The shift in fluorescence indicating amount of 9.3/L6 sFvIg binding to H3347 cells was less than the shift observed for cell surface expression of 2e12 and CD80, but greater than the shift observed for the 9.3 clones.

**Comparison of costimulatory activities of transfected H3347 tumor cells 30 and 9.3/L6 sFvIg coated tumor cells:** The bispecific sFvIg molecule and cell surface expression of the CD28 sFv on tumor cells were compared as methods of providing costimulatory signals to T cells. Rested 7 day PHA blasts were cocultured for three days with irradiated transfected H3347 tumor cells expressing CD80, the 2e12 sFv, the 9.3 sFv, or no exogenous fusion protein. In addition, dilutions of the 35 9.3/L6 sFvIg bispecific were cocultured with PHA blasts alone or with PHA blasts

and untransfected tumor cells. The results of this experiment, shown in Table 2, demonstrate similar levels of T cell proliferation generated by cell surface expressed CD80, 2e12, or 9.3.

A proliferative response was also observed using the bispecific fusion protein 5 alone at high concentrations (non-antigen specific stimulation), or at low concentrations so long as tumor cells were also present (antigen specific targeting of stimulation). These results indicate that the bispecific molecule can function as a costimulatory molecule at a level comparable to that of cell surface expressed sFv or native ligand. This has important implications for the soluble molecule approach to 10 tumor immunotherapy as an alternative to *ex vivo* culture and gene therapy. The results also indicate that the CD28 specific sFvs may be more efficient at costimulation than the CD80 molecule at equivalent cell densities, and that cell surface expression of sFvs may be another promising approach for stimulating tumor specific immune responses.

15 The data shows that expression of the ligands for CD28 and CTLA-4 by tumor cells enhances their immunogenicity and promotes both CD4<sup>+</sup> and CD8<sup>+</sup> T cell mediated tumor rejection under the appropriate conditions (Chen L, et al. Tumor immunogenicity determines the effect of B7 costimulation on T cell-mediated tumor immunity. *J Exp Med* 1994; **179**: 523-532; Chen L, et al. Costimulation of antitumor 20 immunity by the B7 counter-receptor for the T lymphocyte molecules CD28 and CTLA-4. *Cell* 1992; **71**: 1093-1102; Ramarathinam K, et al. T cell costimulation by B7/BB1 induces CD8 T cell-dependent tumor rejection: an important role of B7/BB1 in the induction, recruitment, and effector function of antitumor T cells. *J Exp Med* 1994; **179**: 1205-1214; Johnston JV, et al. B7-CD28 costimulation unveils the 25 hierarchy of tumor epitopes recognized by major histocompatibility complex class I-restricted CD8+ cytolytic T lymphocytes. *J Exp Med* 1996; **183**: 791-800; Baskar S, et al. Constitutive expression of B7 restores immunogenicity of tumor cells expressing truncated major histocompatibility complex class II molecules. *Proc Natl Acad Sci USA* 1993; **90**: 5687-5690; Levitsky HI, et al. Immunization with granulocyte- 30 macrophage colony-stimulating factor-transduced, but not B7-1-transduced, lymphoma cells primes idiotype-specific T cells and generates potent systemic antitumor immunity. *J Immunol* 1996; **156**: 3858-3865). Artificial adhesion receptors were constructed on the cell surface using the 9.3 and 2e12 sFvIgs fused to the CD80 cytoplasmic and transmembrane domains. The data demonstrates that transfection of 35 the cell surface forms of the two different CD28 sFvIgs into H3347 human tumor cells

causes them to generate significant costimulatory signals to resting T cell blasts *in vitro*. The data also demonstrates costimulation using untransfected tumor cells coated with a single chain bispecific  $\alpha$ CD28- $\alpha$ L6 sFvIg fusion protein.

5 This approach to triggering tumor specific immune responses resulted in similar levels of T cell proliferation *in vitro*.

Table 2.

Comparison of costimulatory activities of transfected tumor lines with 9.3/L6 SFvIg coated cells.

H3347 Clone	1:5	1:25	1:100		
9.3 4-1 b1	102	37	11.7		
9.3 6-4 a4	95.3	35.3	8.8		
B7-112	65.4	26.4	5.2		
2e12 3-2/3c	117	73.6	28		
<b>9.3/L6</b>	<b>+H3347</b>	<b>no H3347 cells</b>			
0.5 $\mu$ g/ml	144.7	69.2			
0.1 $\mu$ g/ml	115	9.6			
0.05 $\mu$ g/ml	97.8	4			
<b>H3347 untransfected</b>	<b>cells washed 3x</b>				
9.3/L6, 1 $\mu$ g/ml	98				
media	2.6				
Blasts alone	1.4				
H3347 alone	1.9				

10

In Table 2, rested 7-day PHA blasts were cocultured for three days with irradiated transfected H3347 tumor cells expressing CD80 (B7-112), the 2e12 sFv (2e12 3-2/3c), the 9.3 sFv (9.3 4-1b1 or 9.3 6-4 a4), or untransfected cells, at E:T ratios of 1:5, 1:25, or 1:100. Alternatively, the 9.3/L6 sFvIg bispecific was incubated

in solution at 0.5  $\mu$ g/ml, 0.1  $\mu$ g/ml and 0.05  $\mu$ g/ml with PHA blasts or with blasts and tumor cells (untransfected) at an E:T ratio of 1:5. The bispecific protein was also preincubated with untransfected tumor cells at 1  $\mu$ g/ml, washed once or three times, and cocultured with the PHA blasts at an E:T ratio of 1:5. Cultures were pulsed 5 during the last 6 hours of the three day assay with [ $^3$ H]-thymidine. Results are tabulated as cpm-incorporated  $\times 10^{-3}$ . Each total is the mean of triplicate samples, and SEM  $\pm$  6% for all data.

What is claimed is:

1. A modified sFv molecule which mediates adhesion between lymphocytes or lymphocytes and non-lymphocytic cells, the sFv molecule comprising:
  - (a) a variable region which recognizes and binds an adhesion receptor and
  - (b) at least a portion of the transmembrane region of an adhesion receptor.
2. A modified sFv molecule which mediates adhesion between lymphocytes or lymphocytes and non-lymphocytic cells, the sFv molecule comprising:
  - (a) a variable region of an adhesion receptor,
  - (b) a linker region, and
  - (c) at least a portion of the transmembrane region of an adhesion receptor, the linker connecting the variable region to at least a portion of the transmembrane region.
3. A modified sFv molecule which mediates adhesion between lymphocytes or lymphocytes and other non-lymphocytic cells, the sFv molecule comprising a variable region which recognizes and binds the CD28 receptor, a Fc region, and at least a portion of the transmembrane region of the B7 receptor.
4. The modified sFv molecule of claim 1 further comprising a Fc region, the Fc region connecting the variable region to the transmembrane region.
- 25 5. A complex comprising the modified sFv molecule of claim 1 and a cell, the modified sFv molecule joined to the cell through the transmembrane region of the modified sFv molecule of claim 1.
6. The sFv molecule of claim 3 designated 2e12sFv-hIgG1(Fc-) fusion protein.
- 30 7. The sFv molecule of claim 1 or 2, wherein the first or second CD antigen is selected from a group consisting of CD1, CD2, CD3/TCR, CD4, CD5, T12, CD7, CD8, CD9, CD10, CD11, CD13, CD14, CD15, CD18, CD19, CD20, CD21, CD22, CD23, CD24, CD25, CD26, CD27, CD28, CD29, CD30, CD31, CDw32, CD33, CD34, CD35, CD37, CD38, CD40, CD41, CD43, CD44, CD45, CD46, CD48, CD49, CDw50, CD51, CDw52, CD53, CD54, CD55, CD56,

CD57, CD58, CD59, CDw60, CD61, CD62, CD63, CD64, CDw65, CD66, CD67, CD68, CD69, CDw70, CD71, CD72, CD73, CD74, CD75, CD76, CD77, CDw78, CD80, CD86, CTLA4.

5 8. The sFv molecule of claim 1 or 2, wherein the first or second CD antigen is CD28.

9. The sFv molecule of claim 1 or 2, wherein the first or second CD antigen is B7.

10 10. The sFv molecule of claim 1 or 2, wherein the first or second CD antigen is CTLA4.

11. The sFv molecule of claim 1 or 2, wherein the transmembrane region is selected from a group consisting of CD1, CD2, CD3/TCR, CD4, CD5, T12, CD7, CD8, CD9, CD10, CD11, CD13, CD14, CD15, CD18, CD19, CD20, CD21, CD22, CD23, CD24, CD25, CD26, CD27, CD28, CD29, CD30, CD31, CDw32, CD33, CD34, CD35, CD37, CD38, CD40, CD41, CD43, CD44, CD45, CD46, CD48, CD49, CDw50, CD51, CDw52, CD53, CD54, CD55, CD56, CD57, CD58, CD59, CDw60, CD61, CD62, CD63, CD64, CDw65, CD66, CD67, CD68, 20 CD69, CDw70, CD71, CD72, CD73, CD74, CD75, CD76, CD77, CDw78, B7-1 (CD80), B7-2 (CD86).

12. A nucleic acid molecule encoding the amino acid sequence corresponding to the modified sFv of claim 1, 2, or 3.

25 13. A cDNA of claim 12.

14. A plasmid which comprises the cDNA of claim 13.

30 15. A host vector system comprising a plasmid of claim 14 in a suitable host cell.

16. The host vector system of claim 15, wherein the suitable host cell is a bacterial cell.

35 17. The host vector system of claim 15, wherein the suitable host cell is a eucaryotic cell.

18. A method for producing a protein comprising growing the host vector system of claim 17 so as to produce the protein in the host and recovering the protein so produced.

5

19. A method for producing a biologically active sFv molecule in a mammalian cell which comprises:

- (a) transfected the mammalian cell with the plasmid of claim 16;
- (b) culturing the mammalian cell so transfected in step (a); and
- 10 (c) recovering the biologically active sFv molecule so produced by the cultured mammalian cell.

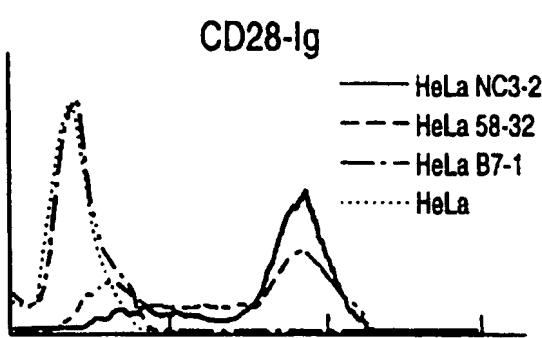
20. The method of claim 19, wherein recovering the biologically active sFv molecule comprises:

- 15 (a) identifying the biologically active sFv molecule by the presence of the variable region; and
- (b) separating the biologically active sFv molecule so identified from molecules without the variable region, so as to recover the biologically active sFv molecule so produced by the cultured mammalian cell.

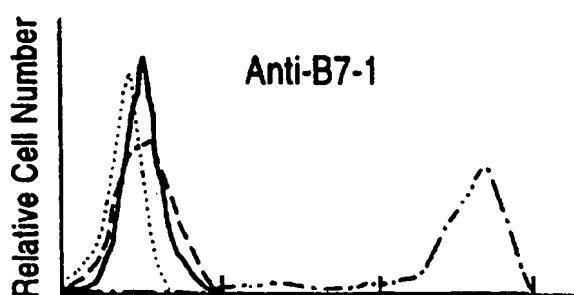
20

21. A biologically active sFv molecule produced by the method of claim 20.

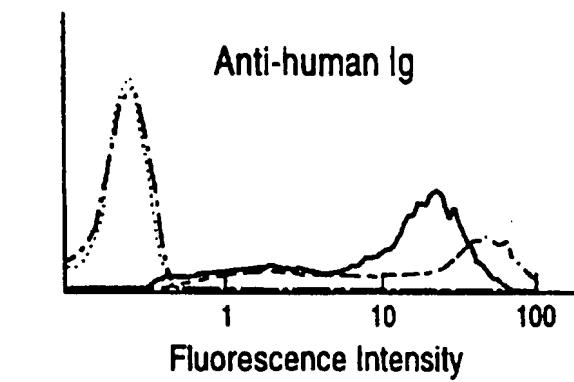
**FIG. IA**



**FIG. IB**

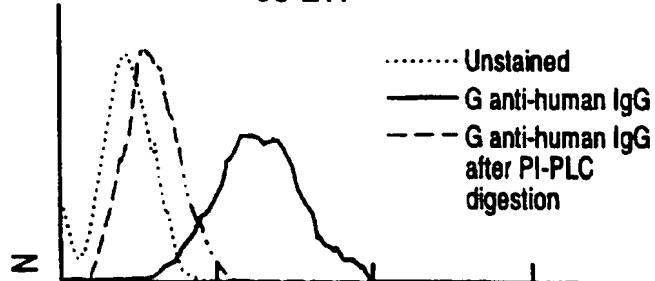


**FIG. IC**



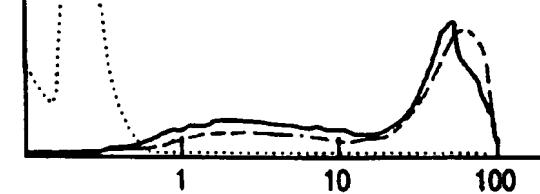
**FIG. 2A**

58-217

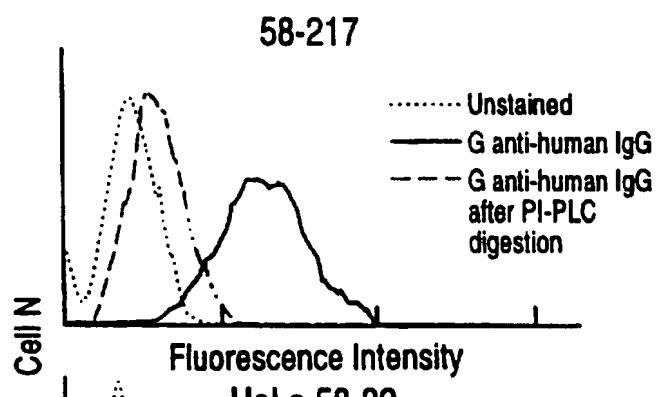


**FIG. 2B**

Fluorescence Intensity  
HeLa 58-32



**FIG. 2A**



**FIG. 2B**

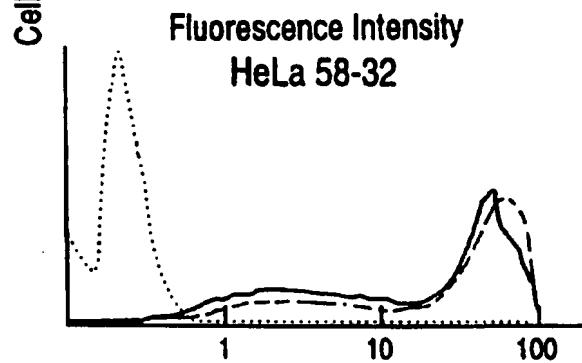


FIG. 3

T-CELL ACTIVATION WITH ANTI-CD28 sFv ON HeLa CELLS

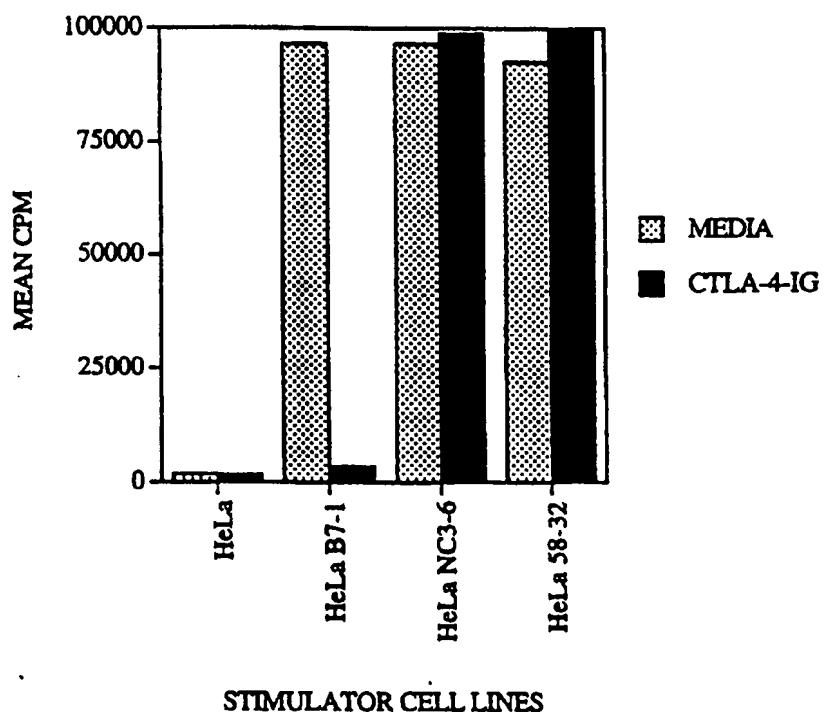
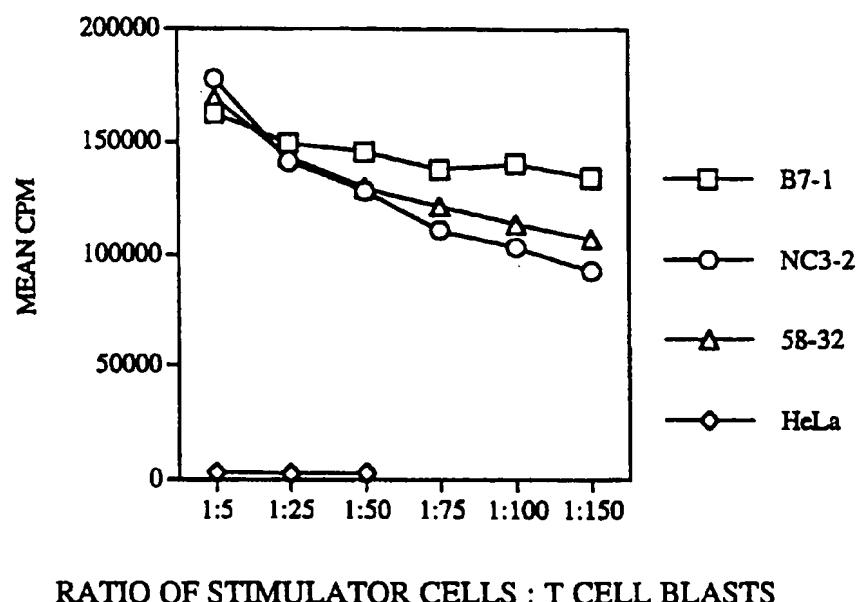
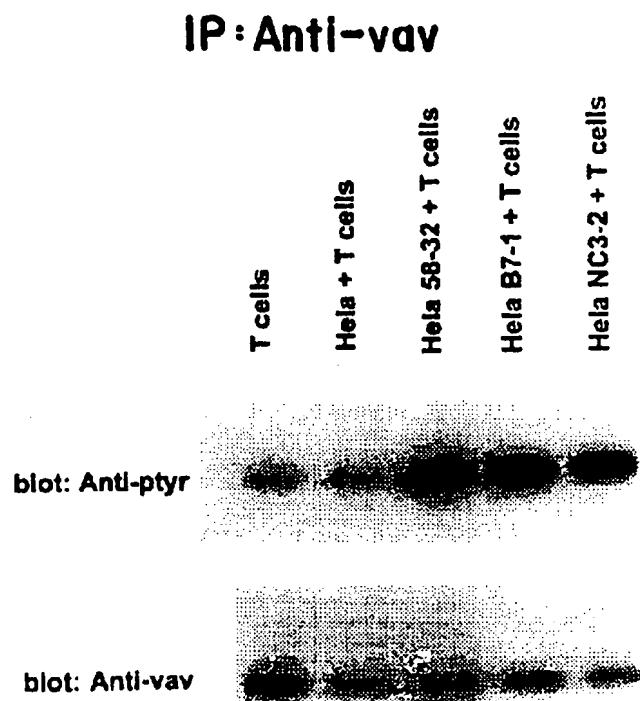


FIG. 4



RATIO OF STIMULATOR CELLS : T CELL BLASTS



**FIG. 5**

FIG. 6

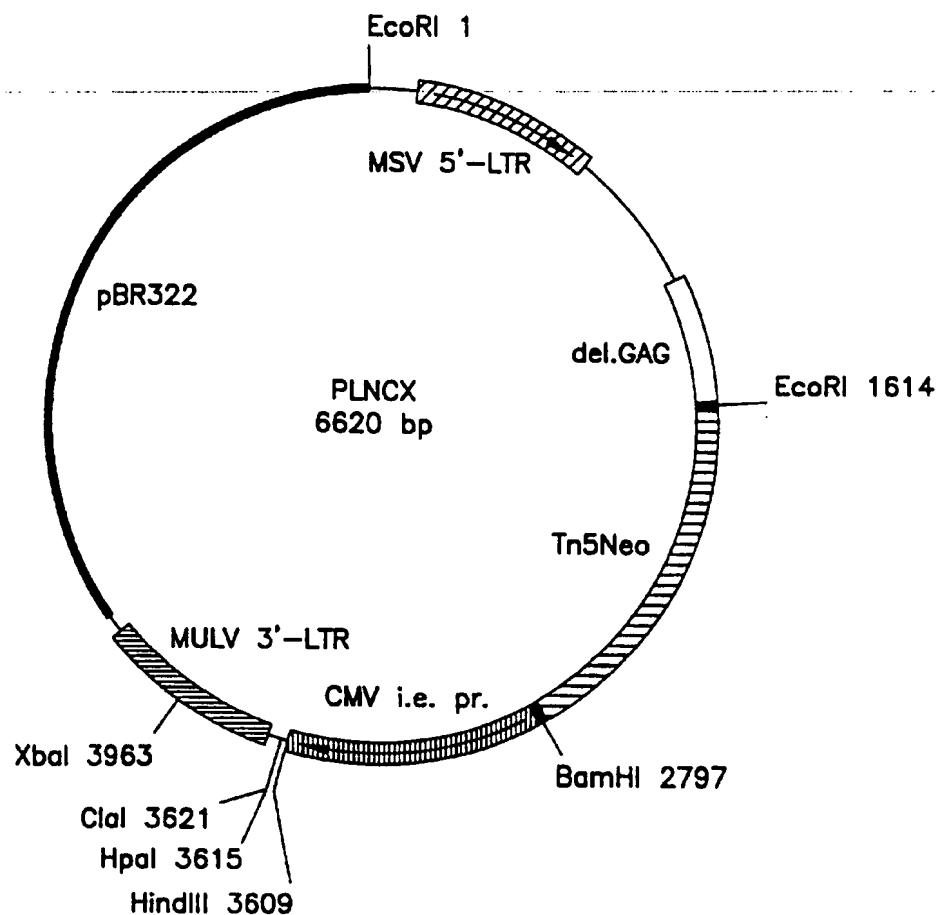
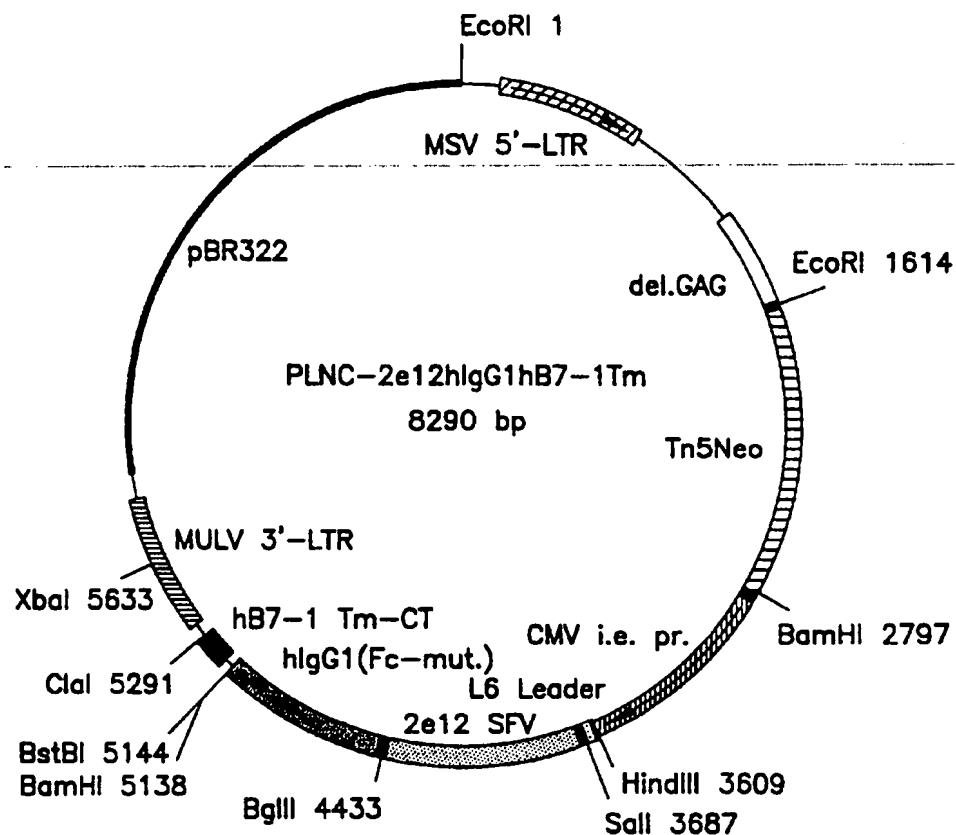


FIG. 7

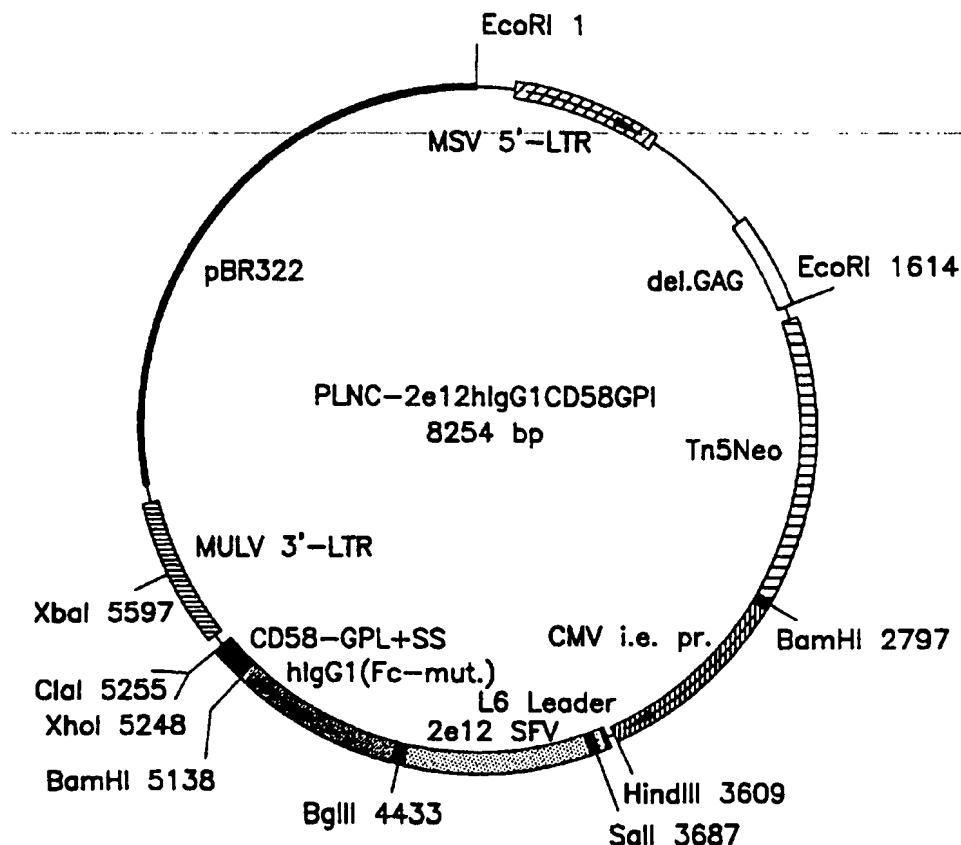


Plasmid name: PLNC-2e12hlgG1hB7-1Tm

Plasmid size: 8290 bp

Comments/References: L6 Leader preceded by 5'-CCACC-3';  
BclI in 2e12 changed to BglII; BamHI-BstBI sequence is:  
5'-GGATCCTTCGAA-3'. hlgG1(Fc-mut) is from P. Linsley.

FIG. 8

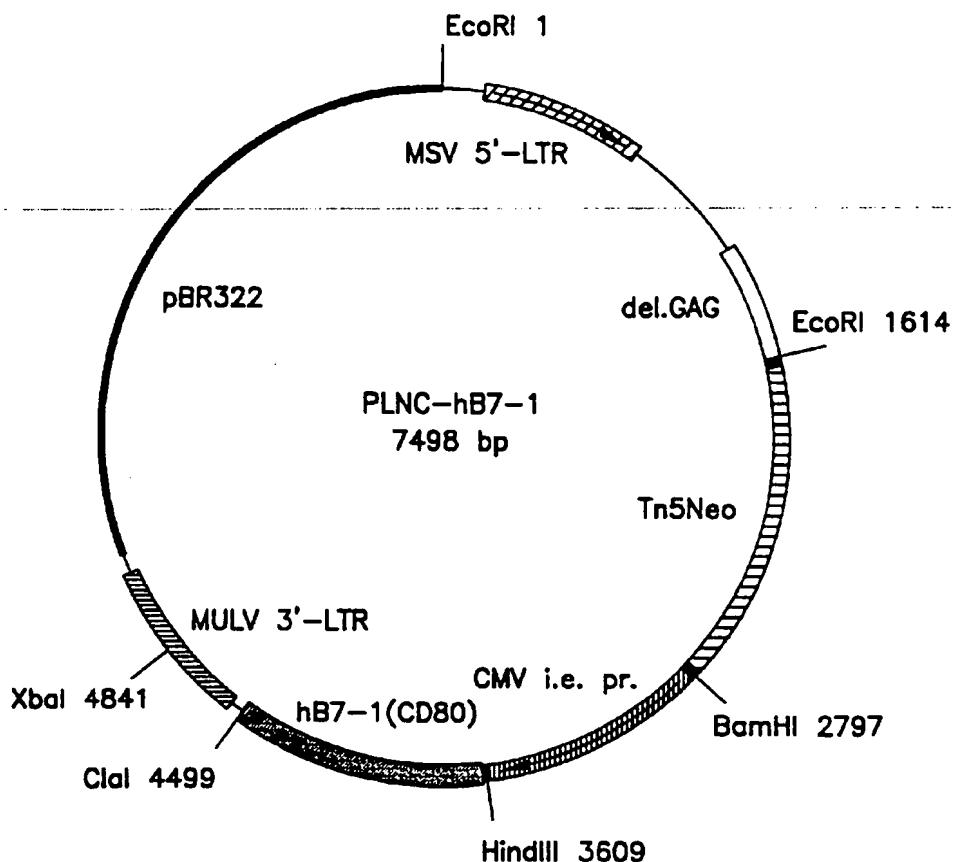


Plasmid name: PLNC-2e12hlgG1CD58GPI

Plasmid size: 8254 bp

Comments/References: CD58GPI-anchor and C-terminal signal sequence is a.a. 207-237 [base 631-739 (incl.)] of cDNA in: Seed, B. 1987. *Nature*, 329:840-842. Xhol-ClaI sequence is 5'-CTCGAGCATCGAT-3'

FIG. 9



Plasmid name: PLNC-hB7-1

Plasmid size: 7498 bp

Comments/References: Human B7-1 (CD80) ATG codon is preceded by 5'-CCACC-3'.

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**FIG. 10A**

NUCLEIC ACID SEQUENCE OF THE 2E12  $\alpha$ Fv INCLUDING THE FC LINKER AND  
B7-1 TRANSMEMBRANE DOMAIN

ATT GTG CTC ACC CAA TCT CCA GCT TCT TTG GCT GTG TCT CTA GGT CAG AGA  
CCC ACC ATC TCC TGC AGA GCC AGT GAA AGT GTT GAA TAT TAT GTC ACA AGT  
TTA ATG CAG TGG TAC CAA CAG AAA CCCA GGA CAG CCA CCC AAA CTC CTC  
ATC TCT GCT GCA TCC AAC GTA GAA TCT GGG GTC CCT GCC AGG TTT AGT GGC  
AGT GGG TCT GGG ACA GAC TTC AGC CTC AAC ATC CAT CCT GTG GAG GAG GAT  
GAT ATT GCA ATG TAT TTC TGT CAG CAA AGT AGG AAG GTT CTT TGG ACG TTC  
GGT GGA GGC ACC AAG CTG GAA ATC AAA CGG GGT GGC GGT GGC TCG GGC GGT  
GGT GGG TCG GGT GGC GGC GGA TCT CAG GTG CAG CTG AAG GAG TCA GGA CCT  
GGC CTG GTG GCG CCC TCA CAG AGC CTG TCC ATC ACA TGC ACC GTC TCA GGG  
TTC TCA TTA ACC GGC TAT GGT GTA AAC TGG GTT CGC CAG CCT CCA GGA AAG  
GGT CTG GAG TGG CTG GGA ATG ATA TGG GGT GAT GGA AGC ACA GAC TAT AAT  
TCA GCT CTC AAA TCC AGA CTG AGC ATC ACC AAG GAC AAC TCC AAG AGC CAA  
GTT TTC TTA AAA ATG AAC AGT CTG CAA ACT GAT GAC ACA GCC AGA TAC TAC  
TGT GCC AGA GAT GGT TAT AGT AAC TTT CAT TAC TAT GTT ATG GAC TAC TGG  
GGT CAA GGA ACC TCA GTC ACC GTC TCC TCT GAT CCG GAG CCC AAA TCT TGT  
GAC AAA ACT CAC ACA TGC CCA CCG TGC CCA GCA CCT GAA TTC GAG GGT GCA  
CCG TCA GTC TTC CTC CCC CCA AAA CCC AAG GAC AAC CTC ATG ATC TCC  
CGG ACC CCT GAG GTC ACA TGC GTG GTG GAC GTG AGC CAC GAA GAC CCT  
GAG GTC AAG TTC AAC TGG TAC GTG GAC GCC GTG GAG GTG CAT AAT GCC AAG  
ACA AAG CCG CGG GAG GAG CAG TAC AAC AGC ACG TAC CGG GTG GTC AGC GTC  
CTC ACC GTC CTG CAC CAG GAC TGG CTG AAT GGC AAG GAG TAC AAG TGC AAG  
GTC TCC AAC AAA GCC CTC CCA GCC CCC ATC GAG AAA ACC ATC TCC AAA GCC  
AAA GGG CAG CCC CGA GAA CCA CAG GTG TAC ACC CTG CCC CCA TCC CGG GAT  
GAG CTG AAC AAG AAC CAG GTC AGC CTG ACC TGC CTG GTC AAA GGC TTC TAT

## FIG. 10B

CCC AGC GAC ATC GCC GTG GAG TGG GAG AGC AAT GGG CAG CCG GAG AAC AAC  
TAC AAG ACC ACG CCT CCC GTG CTG GAC TCC GAC GGC TCC TTC TTC CTC TAC  
AGC AAG CTC ACG GTG GAC AAG AGC AGG TGG CAG CAG GGG AAC GTC TTC TCA  
TGC TCC GTG ATG CAT GAG GCT CTG CAC AAC CAC TAC ACG CAG AAG AGC CTC  
TCC CTG TCT CCG GGT AAA AAC CTG CTC CCA TCC TGG GCC ATT ACC TTA ATC  
TCA GTA AAT GGA ATT TTT GTC ATA TGC TGC ACC TAC TGG TTT GCC CCA

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## FIG. II A

NUCLEIC ACID SEQUENCE OF THE 2E12 sFv INCLUDING THE FC LINKER AND  
GPI TRANSMEMBRANE DOMAIN

ATT GTG CTC ACC CAA TCT CCA GCT TCT TTG GCT GTG TCT CTA GGT CAG AGA  
CCC ACC ATC TCC TGC AGA GCC AGT GAA AGT GTT GAA TAT TAT GTC ACA AGT  
TTA ATG CAG TGG TAC CAA CAG AAA CCCA GGA CAG CCA CCC AAA CTC CTC  
ATC TCT GCT GCA TCC AAC GTA GAA TCT GGG GTC CCT GCC AGG TTT AGT GGC  
AGT GGG TCT GGG ACA GAC TTC AGC CTC AAC ATC CAT CCT GTG GAG GAG GAT  
GAT ATT GCA ATG TAT TTC TGT CAG CAA AGT AGG AAG GTT CTT TGG ACG TTC  
GGT GGA GGC ACC AAG CTG GAA ATC AAA CGG GGT GGC GGT GGC TCG GGC GGT  
GGT GGG TCG GGT GGC GGA TCT CAG GTG CAG CTG AAG GAG TCA GGA CCT  
GGC CTG GTG GCG CCC TCA CAG AGC CTG TCC ATC ACA TGC ACC GTC TCA GGG  
TTC TCA TTA ACC GGC TAT GGT GTA AAC TGG GTT CGC CAG CCT CCA GGA AAG  
GGT CTG GAG TGG CTG GGA ATG ATA TGG GGT GAT GGA AGC ACA GAC TAT AAT  
TCA GCT CTC AAA TCC AGA CTG AGC ATC ACC AAG GAC AAC TCC AAG AGC CAA  
GTT TTC TTA AAA ATG AAC AGT CTG CAA ACT GAT GAC ACA GCC AGA TAC TAC  
TGT GCC AGA GAT GGT TAT AGT AAC TTT CAT TAC TAT GTT ATG GAC TAC TGG  
GGT CAA GGA ACC TCA GTC ACC GTC TCC TCT GAT CCG GAG CCC AAA TCT TGT  
GAC AAA ACT CAC ACA TGC CCA CCG TGC CCA GCA CCT GAA TTC GAG GGT GCA  
CCG TCA GTC TTC CTC TTC CCC CCA AAA CCC AAG GAC AAC CTC ATG ATC TCC  
CGG ACC CCT GAG GTC ACA TGC GTG GTG GAC GTG AGC CAC GAA GAC CCT  
GAG GTC AAG TTC AAC TGG TAC GTG GAC GCC GTG GAG GTG CAT AAT GCC AAG  
ACA AAG CCG CGG GAG GAG CAG AAC AGC ACG TAC CGG GTG GTC AGC GTC  
CTC ACC GTC CTG CAC CAG GAC TGG CTG AAT GGC AAG GAG TAC AAG TGC AAG  
GTC TCC AAC AAA GCC CTC CCA GCC CCC ATC GAG AAA ACC ATC TCC AAA GCC  
AAA GGG CAG CCC CGA GAA CCA CAG GTG TAC ACC CTG CCC CCA TCC CGG GAT  
GAG CTG ACC AAG AAC CAG GTC AGC CTG ACC TGC CTG GTC AAA GGC TTC TAT

**FIG. 11B**

CCC AGC GAC ATC GCC GTG GAG TGG GAG AGC AAT GGG CAG CCG GAG AAC AAC  
TAC AAG ACC ACG CCT CCC GTG CTG GAC TCC GAC GGC TCC TTC TTC CTC TAC  
AGC AAG CTC ACC GTG GAC AAG AGC AGG TGG CAG CAG GGG AAC GTC TTC TCA  
TGC TCC GTG ATG CAT GAG GCT CTG CAC AAC CAC TAC ACG CAG AAG AGC CTC  
TCC CTG TCT CCG GGT AAA TAT GCA CTT ATA CCC ATA CCA TTA GCA GTA ATT  
ACA ACA TGT ATT GTG CTG TAT ATG AAT GTT CTT

## FIG.12A

1	GTCGACATTG	TGCTCACCCA	ATCTCCAGCT	TCTTTGGCTG	TGTCTCTAGG
51	TCAGAGAGCC	ACCATCTCCT	GCAGAGCCAG	TGAAAGTGT	GAATATTATG
101	TCACAAGTTT	AATGCAGTGG	TACCAACAGA	AACCAGGACA	GCCACCCAAA
151	CTCCTCATCT	CTGCTGCATC	CAACGTAGAA	TCTGGGTCC	CTGCCAGGTT
201	TAGTGGCAGT	GGGTCTGGGA	CAGACTTCAG	CCTCAACATC	CATCCTGTGG
251	AGGAGGATGA	TATTGCAATG	TATTCTGTGTC	AGCAAAGTAG	GAAGGTTCT
301	TGGACGTTCG	GTGGAGGCAC	CAAGCTGGAA	ATCAAACGGG	GTGGCGGTGG
351	CTCGGGCGGT	GGTGGGTCGG	GTGGCGGCAG	ATCTCAGGTG	CAGCTGAAGG
401	AGTCAGGACC	TGGCCTGGTG	GCGCCCTCAC	AGAGCCTGTC	CATCACATGC
451	ACCGTCTCAG	GGTTCTCAIT	AACCGGCTAT	GGTGTAAACT	GGGTTCGCCA
501	GCCTCCAGGA	AAGGGTCTGG	AGTGGCTGGG	AATGATATGG	GGTGATGGAA
551	GCACAGACTA	TAATTCAAGCT	CTCAAATCCA	GAATGAGCAT	CACCAAGGAC
601	AACTCCAAGA	GCCAAGTTTT	CTTAAAAATG	AACAGTCTGC	AAACTGATGA
651	CACAGCCAGA	TACTACTGTG	CCAGAGATGG	TTATAGTAAC	TTTCATTACT
701	ATGTTATGGA	CTACTGGGT	CAAGGAACCT	CAGTCACCGT	CTCCTCTGAT
751	CA				

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## FIG.12B

(Linear) (Six Base) Map of: Rgamma. check: 7812 from: 1 to: 767

LOCUS HUIGGI MUT 767 BP DS-DNA

DEFINITION

ACCESSION

KEYWORDS

SOURCE

BASE COUNT

185 A 253 C 207 G 122 T 0 OTHER

with 139 enzymes: \*

B	B
s	s
P	p
BB BB	B1
as ss	a2
mt ap	n8
HY WE	I6
II II	II
GGATCCGGAGCCCCAAATCTTGTGACAAAACTCACACATGCCACCGTGCCCAGCACCTGA	
1	----- 60

b D P E P K S C D K T H T C P P C P A P E

B	B
s	s
A	p
1	1H
W	B2g
4	b8i
4	s6A
I	III
ATTCGAGGGTGCACCGTCAGTCTTCCCTCTTCCCCCAAGGACACCCCTCATGAT	
61	----- 120

b F E G A P S V F L F P P K P K D T L M I

B	B
s	s
u	MN
3	ss
6	lp
I	II
CTCCCGGACCCCTGAGGTACATGCGTGGTGGACCTGAGCCACGAAGACCCCTGAGGT	
121	----- 180

b S R T P E V T C V V V D V S H E D P E V

B	N
s	ss
a	D pa
A	s Bc
I	a II
CAAGTTCAACTGGTACGTGGACGCCCTCCAGGTCCATAATGCCAAGACAAAGCCGGGA	
181	----- 240

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FIG.12C

b K F N W Y V D G V E V H N A K T K P R E -

B		B		E
s		s		C
e		a		o
R		A		N
I		I		I

GGAGCAGTACAACAGCACGTACCGGGTGGTCAGCGTCTCACCGTCTGCACCAGGACTG

241-----300

b E Q Y N S T Y R V V S V L T V L H Q D W -

B		M
s		m
a		e
I		I

GCTGAATGGCAAGGAGTACAAGTCCAAGGTCTCCAACAAAGCCCTCCAGCCCCATCGA

301-----360

b L N G K E Y K C K V S N K A L P A P I E -

B		
s		
p		
D	1	
A	r	4
v	d	0
a	I	7
I	I	I

GAAAACCATCTCAAAGCCAAAGGGCAGCCCCGAGAACCAACAGGTGTACACCTGCCCCC

361-----420

b K T I S K A K G Q P R E P Q V Y T L P P -

DS		B
A S	re	s
v m	dx	p
a a	IA	M
I I	II	I

ATCCCGGGATGAGCTGACCAAGAACCAAGGTCAAGCTGACCTGCCTGGTCAAAGGTTCTA

421-----480

b S R D E L T K N Q V S L T C L V K G F Y -

MD		B
ss		s
la		r
II		D
		I

TCCCAAGCGACATCGCCGTGGAGTGGAGAGCAATGGCAGCCGGAGAACAACTACAAGAC

481-----540

b P S D I A V E W E S N G Q P E N N Y K T -

B		B
s		s
p	f	m
G	C	e
I	I	I

CACGCCTCCCGTGTGGACTCCGACGGCTCCTCTTCTACAGCAAGCTCACCGTGG

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## FIG.12D

541-----

600

B T P P V L D S D G S F F L Y S K L T V D -

B	X	B	N
b	m	s	s
s	n	g	i
I	I	I	I

CAAGAGCAGGTGGCAGCAGGGAAACGTCTCTCATGCTCCGTGATGCATGAGGCTCTGCA

601-----

660

b K S R W Q Q G N V F S C S V M H E A L H -

ES	BBG
aa	EEssdN
rp	aariia
II	egeeeie
	IIIIII

CAACCACTACACGAGAAGAGCCTCTCCCTGTCTCCGGTAAATGAGTGCACGGCCGGC

661-----

720

b N H Y T Q K S L S L S P G K \* V R R P A -

B		
s		
p		
B	B1	B
s A S	a2	s B M X
r v m	n8	I s s b
B a a	I6	E I I a
I I I	II	I I I I

AAGCCCCCTCCCCGGCTCTCGCGGTGCGACGAGGATGCTTCTAGA

721-----

767

b S P A P R A L A V A R G C F \* -

Enzymes that do cut:

Alw44I	AlwNI	ApoI	AvaI	BamHI	BanII	BbsI	BsaI
BsaAI	BsaWI	BseRI	BsgI	BsiI	BsiEI	Bsp1286I	Bsp1407I
BspKI	BspGI	BspHI	BspMI	BsrBI	BsrDI	BsrFI	BatYI
Bsu36I	DrdI	DrdII	DsaI	EaeI	EagI	EarI	EcoNI
EcoRI	GdIII	HgiAI	MmeI	MslI	NaeI	NsiI	NspI
NspBII	SacII	SapI	SexIA	SfcI	SmaI	StyI	XbaI
XbaI							

## FIG.12E

Enzymes that do not cut:

AatII	AccI	AflIII	AfI1II	AgeI	Apal	ApaBI	AsCI
Asel	AvrII	BaeI	BanI	Bce83I	BcgI	BcgI	BclI
BglII	BglIII	BpmI	BPUI01	Bpu102I	BsaBI	BsaHI	HsmI
BssHII	Bst1107I	BstEII	BstXI	ClaI	DraI	DraIII	Eam1105I
EciI	Eco47III	Eco57I	EcoO109I	EcoRV	Esp3T	FseI	FspI
HaeI	HaeII	HqIEII	HincII	HindIII	PhaI	KpnI	MluI
MscI	MunI	NarI	NcoI	NdeI	NheI	NotI	NruI
NspV	PacI	Pf12308I	Pf1MI	PmeI	PmlI	PpuMI	PshAI
Psp1406I	PstI	PvuI	PvuII	RleAI	RsrII	SalI	ScaI
SfiI	SgrAI	SnaBI	SpeI	SphI	SrfI	Sse8387I	SspI
SstI	StuI	SunI	Swal	TaqII	TaqII	Tth111I	Tth111II
XcmI	XhoI						

## FIG.12F

## B7-1 TRANSMEMBRANE DOMAIN

AAC CTG CTC CCA TCC TGG GCC ATT ACC TTA ATC TCA GTA AAT  
 GGA ATT TTT GTC ATA TGC TGC CTG ACC TAC TGG TTT GCC CCA

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## FIG.12G

1 GCCCGACGAGCCATGGITGCTGGCAGCGACGGGGCGGGCCCTGGGGTCTCAGCGTGGCTGC  
 1 MetValAlaGlySerAspAlaGlyArgAlaLeuGlyValLeuSerValValCys

67 CTGCTGCACTGCTTGGTTCATCAGCTGTTTCCCACAAATATGGTGTGTATGGAAAT  
 19 LeuLeuHisCysPheGlyPheIleSerCysPheSerGlnGlnIleTyrGlyValValTyrGlyAsn

133 GTAACTTCCATGTACCAAGCAATGTGCCTTAAAAGAGGTCTATGGAAAAAAACAAAGGATAAA  
 41 ValThrPheHisValProSerAsyValProLeuLysGluValLeuTrpLysLysGlnLysAspLys  
 CHO---

199 GTTGCAGAACTGGAAAATTCTGAATTACAGCTTCTCATCTTTAAAAATAGGGTTATTTAGAC  
 63 ValAlaGluLeuGluAsnSerGluPheArgAlaPheSerSerPheLysAsnArgValTyrLeuAsp

266 ACTGTGTCAGGTAGCCTCACTATCTACAACTAACATCATCAGATGAAGATGAGTATGAAATGGAA  
 85 ThrValSerGlySerLeuThrIleTyrAsnLeuThrSerSerAspGluAspGluTyrGluMetGlu  
 ---CHO---

331 TCGCCAAATATTACTGATACCAGAAGTTCTTCTTATGTGCTTGAGTCTCTCCATCTCCCACA  
 107 SerProAsnIleThrAspThrMetLysPhePheLeuTyrValLeuGluSerLeuProSerProThr  
 ---CHO---

397 CTAACTTGTGCAATTGACTAATGGAAGCATTGAAGTCCAATGCATGATAACCAGAGCATTACAACAGC  
 129 LeuThrCysAlaLeuThrAsnGlySerIleGluValGlnCysMetIleProGluHisTyrAsnSer  
 ---CHO---

463 CATCGAGGACTTATAATGTAACATGGGATTGCTCTATGGAGCAATGTAACGTAACCTAACAG  
 151 HisArgGlyLeuIleMetTyrSerTrpAspCysProMetGluGlnCysLysArgAsnSerThrSer

529 ATATATTTAAGATGGAAAATCATCTTCCACAAAAAAACAGTGTACTCTTAGCAATCCATTATTT  
 173 IleTyrPheLysMetGluAsnAspLeuProGlnLysIleGlnCysThrLeuSerAsnProLeuPhe

595 AATACAACATCATCAATCATTGACAACCTGTATCCCAAGCAGCGGTCTTCAAGACACAGATAT  
 195 AsnThrThrSerSerIleIleLeuThrThrCysIleProSerSerGlyHisSerArgHisArgTyr  
 ---CHO---

661 GCACTTATACCCATACCATTAGCAGTAATTACAACATGTATTGTGCTGTATGAAATGTTCTTAA  
 217 AlaLeuIleProIleProLeuAlaValIleThrThrCysIleValLeuTyrMetAsnValLeuEnd

727 TTGAGAAGACAATTCTCATTTAGGTATTCTGAAATGTGACAGAAAACCAGACACAACCAACT

793 CCAATTGATTGTAACAGAAGATGAACACAACAGCATAACTAAATTATTTAAAAACTAAAAAGCC

859 ATCTGATTCTCATTT

The sites of potential N-linked glycosylation are denoted by the symbol -CHO-; the hydrophobic carboxyl terminus is underscored.

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## FIG. 13 9.3 sFv NUCLEOTIDE AND DEDUCED AMINO ACID SEQUENCE

LEADER

HindIII M E S D T L L L W V L I L W V P G S  
**AAGC**TATGGAGTCAGACACACTCCTGCTATGGGTGCTGCTGCTCTGGGTTCCAGGCTCC

V<sub>L</sub> →

10

T G D I V L T Q S P A S L A V S L G Q R  
 ACTGGTGACATTGTGCTCACCCAAATCTCCAGCTTGGCTGTGCTCTAGGGCAGAGA

20

CDR1 27 a b c d 30

A T I S C R A S E S V E Y Y V T S L M Q  
 GCCACCATCTCCTGCAGAGCCAGTGAAGGTGAAATATTATGTCACAAGTTAACGAG

40

50 CDR2

W Y Q Q K P G Q P P K L L I F A A S N V  
 TGGTACCAAGCAGAAGCCAGGACAGCCACCCAAA CTCCTCATTTGCTGCATCCAACGTA

60

70

E S G V P A R F S G S G S G T N F S L N  
 GAATCTGGGTCCCTGCCAGGTTAGTGGCAGTGGTCTGGGACAAACCTCAGCCTCAAC

80

90 CDR3

I H P V O E D D V A M Y F C Q Q S R K V  
 ATCCATCCTGTGGACGAGGATGATGTTGCAATGTATTCTGTCAAAGTAGGAAGGTT

100

(GLY, SER),  
 PYT F G G G T K L E I K R A S G G G G  
 CCTAACACGTCGGAGGGGGGACCAAGCTGGAAATAAAACGGGCTTCG GGTGGTGGCGGT  
 "Jk1"

V<sub>H</sub> →

1

S G G G G S G G G S L A Q V Q L K E S  
 TCTGGAGGTGGCGGTTCAAGGCAGGGATCCCTGGCTCAGGTGCAGCTGAAGGAGTC

BamIII

10

20

G P G L V T P S Q S L S I T C T V S G F  
 GGACCTGGCCTGGTGACGCCCTCACAGAGCCTGTCATCACTGTACTGTCCTGGGTT

30 CDR1 40

S L S D Y G V H W V R Q S P G Q G L E C  
 TCATTAAGCGACTATGGTGTTCAT TGGGTTGCCAGTCTCAGGACAGGGACTGGAGTC

50 52 a CDR2 60

L G V I W A G G G T N Y N S A L M S R K  
 CTGGGAGTAATATGGGCTGGTGGAGGGACGAATTATAATTCCGGCTCTCATGTCCAGAAAG

70

80 82 a b

S I S K D N S K G Q V F L K M K S L Q A  
 AGCATCAGCAAAGACAACCTCAAGGGCCAAGTTCTTAAAGAAGAGTCTGCAAGCT

90 CDR3 100 a b c d

D D T A V Y Y C A R D K G Y S Y Y Y S M  
 GATGACACAGCCGTGTATTACTGTGCCAGAGATAAGGGATACTCCTATTACTATTCTATG

110

D Y W G Q G T S V T V S S D  
 GACTACTGGGGTCAAGGAACCTCAGTCACCGTCTCCCTGATCA

JH4\* Bcl1

1 2 3 4

—97.4

—69

—46

—30

—21.5

FIG. 14

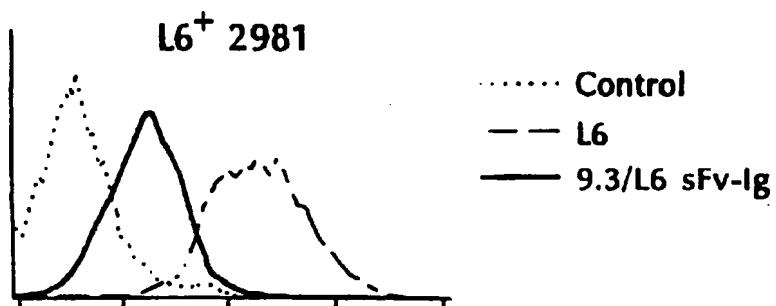
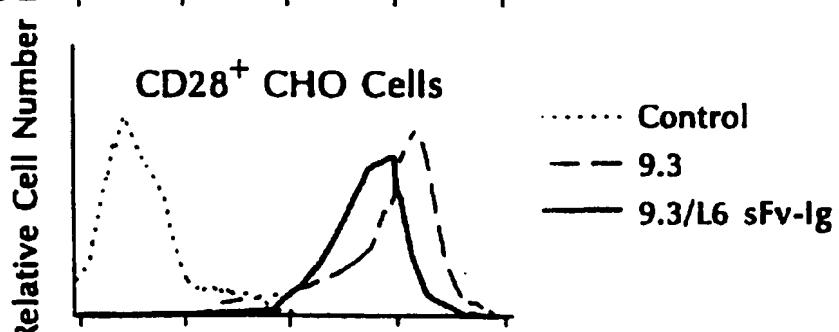
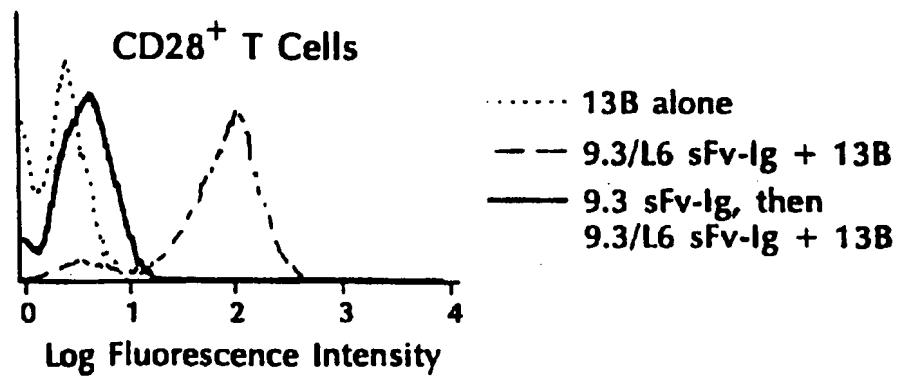
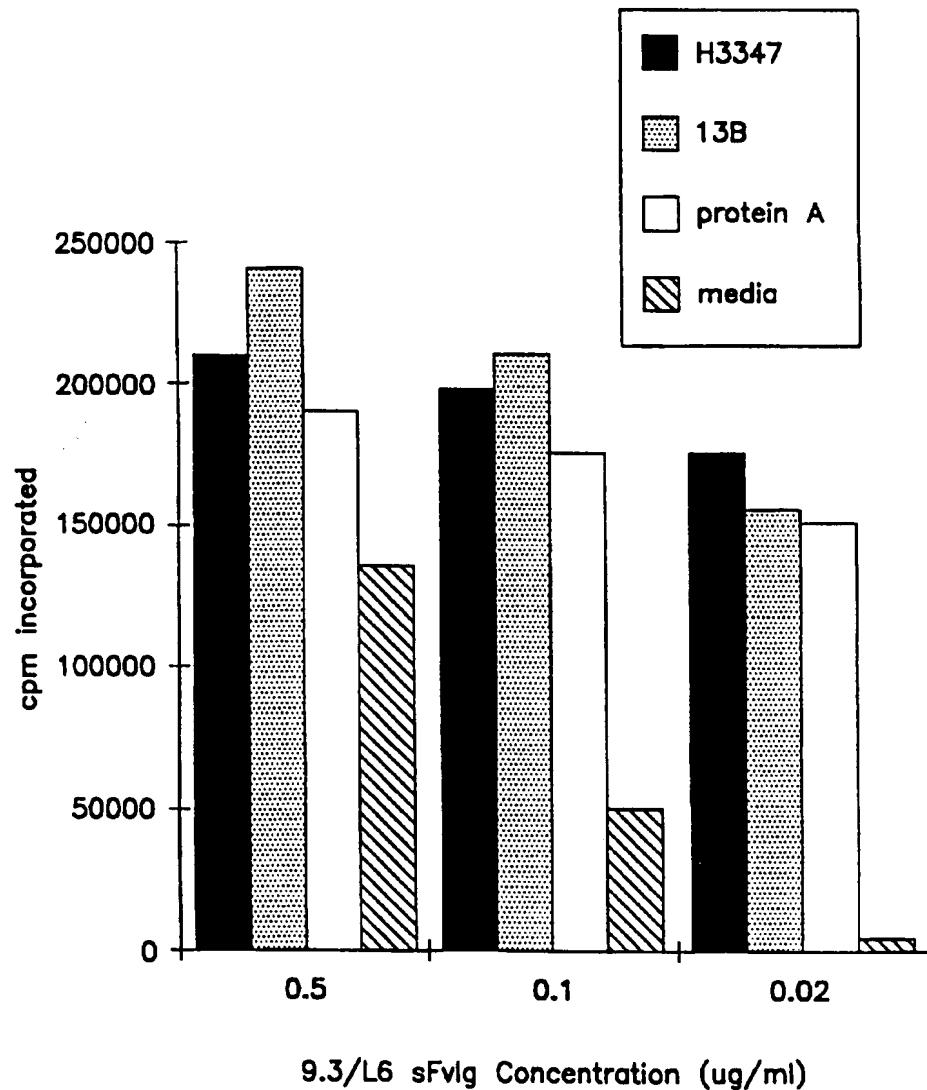
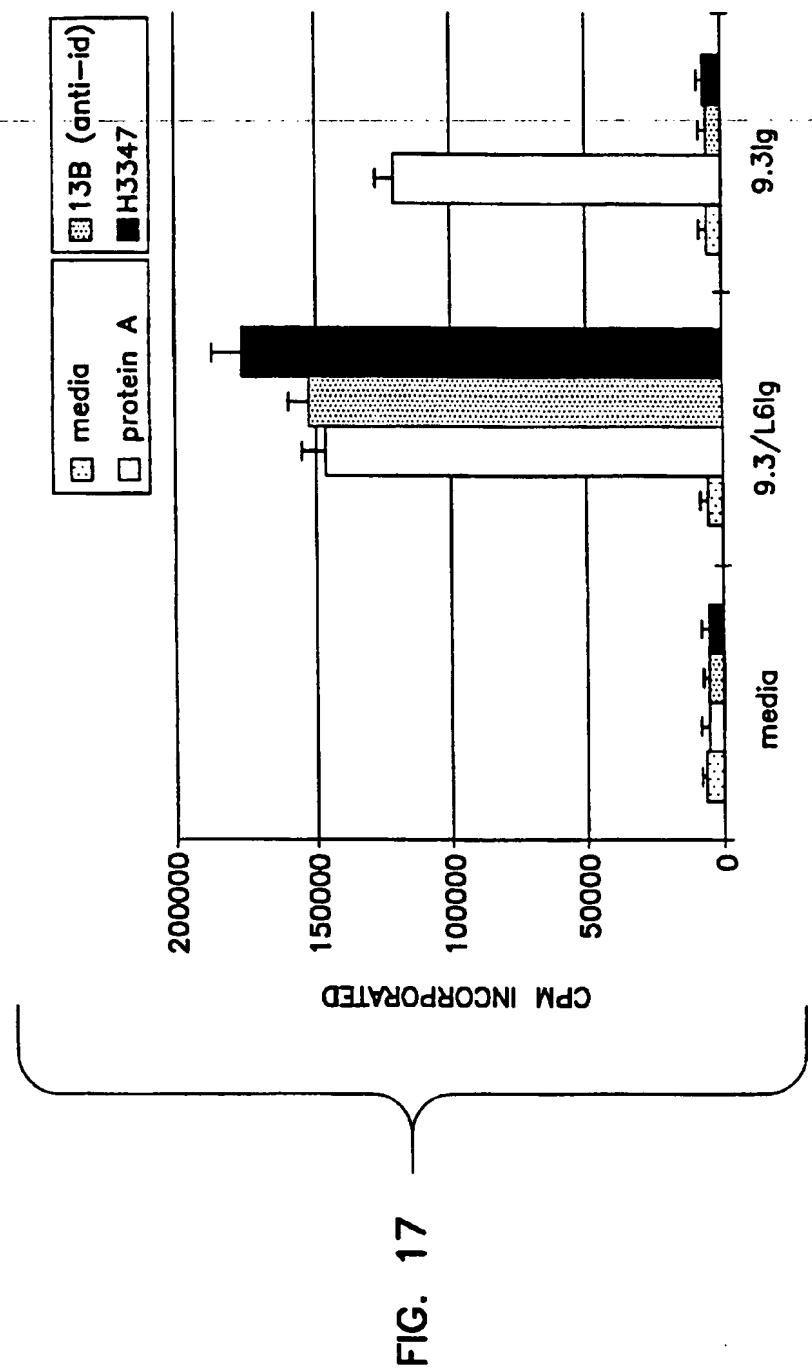
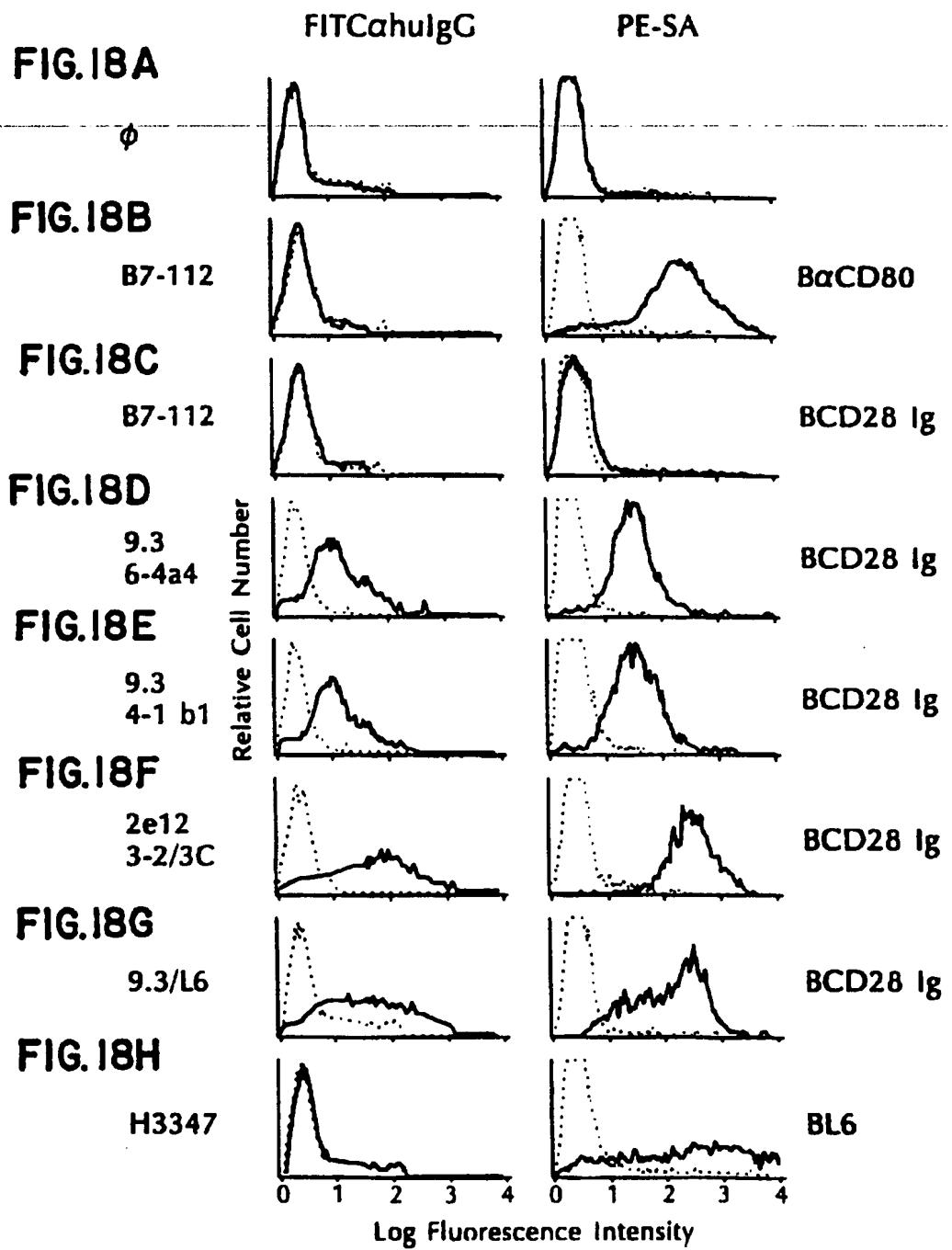
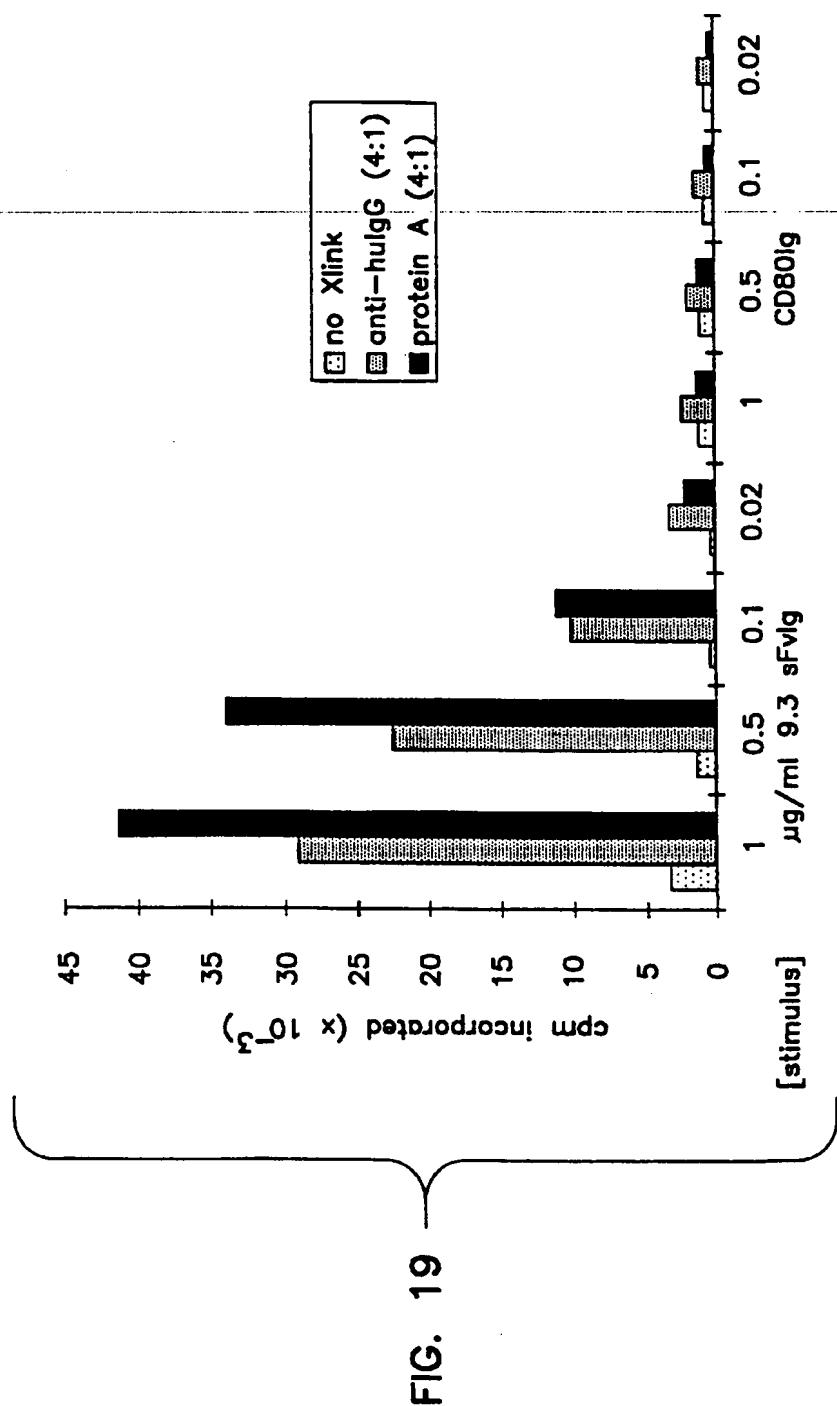
**FIG.15A****FIG.15B****FIG.15C**

FIG. 16









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